

5. I earned a Doctor of Philosophy (PhD) degree in Nutritional Biochemistry (with minors in histology and cell biology) from the University of Missouri in Columbia, Missouri in 1989.
6. From 1991 to 1993, after completing my PhD, I was a Postdoctoral Research Fellow in the Department of Animal Sciences at Purdue University where I researched β -adrenoceptor kinetics and also researched signal transduction in porcine adipose tissue and skeletal muscle.
7. A brief resume of my professional career from 1989 to the present is attached hereto as Exhibit A.
8. I have authored (or coauthored) more than thirty-five articles, including topics concerning leptin technology; many of these articles are listed in my resume of Exhibit A. Some of my more recent leptin articles are listed below:

- * Houseknecht, K.L., and M.E. Spurlock. 2003. The Regulation of Lipid Metabolism in Peripheral Tissues by Leptin, Invited Review, Nutrition Reviews International 16:83-96;
- * Ajuwon, K.M., J.L. Kuske, D.M. Ragland, L. Adeola, D.L. Hancock, D.B. Anderson, and M.E. Spurlock. 2003. The Regulation of Igf-1 by Leptin in the Pig Is Tissue Specific and Independent of Changes in Growth Hormone, J. Nutr. Biochem. 14(9):522-530;
- * Ajuwon, K.M., J.L. Kuske, D.B. Anderson, D.L. Hancock, K.L. Houseknecht, O. Adeola, and M.E. Spurlock. 2003. Chronic Leptin Administration Increases Serum NEFA in the Pig and Differentially Regulates Ppar Expression in Adipose Tissue, J. Nutr. Biochem. 14(10):576-583;

- * Raman, P., S. S. Donkin, and M. E. Spurlock. 2003. Regulation of Hepatic Glucose Metabolism by Leptin in Pig and Rat Primary Hepatocyte Cultures, Am J Physiol Regul Integr Comp Physiol. 286(1): R206-16;
 - * Jacobi, S., K.M. Ajuwon, T.E. Weber, J.L. Kuske, C.J. Dyer, and M.E. Spurlock. Cloning and Expression of Porcine Adiponectin, and its Relationship to Adiposity, Lipogenesis, and the Acute Phase Response, J. Endo. (in press);
 - * Ajuwon, K. M., S. K. Jacobi, J. L. Kuske, and M. E. Spurlock. 2004. Interleukin-6 and Interleukin-15 Are Selectively Regulated by Lipopolysaccharide in Primary Pig Adipocytes, Am J Physiol. 286(3):R547-53;
 - * Weber, T. E., and M. E. Spurlock. 2004. Leptin Alters Antibody Isotype in The Pig in Vivo, And Prevents Dexamethasone-induced Down-regulation of The Anti-apoptotic Gene, Bcl-xl, in Peripheral Blood Monocytes in Vitro, J. Anim. Sci. (in press);
 - * Wulster-Radcliffe, M. C., J. A. Christian, J. Wang, and M. E. Spurlock. 2004. The Anti-inflammatory Actions of Adiponectin Include the Regulation of Il-6 and Il-10, and a Suppression of Cell Proliferation That Is Associated with Increased Caspase Activity, Biochem. Biophys. Res. Comm. 316:924-929.
9. I am experienced in isolating, purifying, and manipulating DNA sequences, such as porcine, bovine, murine, and human DNA sequences, and am also skilled in experimental procedures of leptin genetics, such as techniques for establishing the impact of leptin on lipid accumulation via processes including lipolysis and lipogenesis, due to my education and work experience relating to DNA sequence and leptin genetics issues over the past twenty-five years.

10. I am a co-inventor of the invention described and claimed in U.S. Patent Application Serial No. 09/932,888 filed on August 20, 2001 and am a co-inventor of the invention defined and claimed in U.S. Serial Application No. 08/692,922, now U.S. Patent No. 6,277,592.
11. The formation of a duplex by nucleic acid hybridization (base pairing between two nucleic acid molecules) is directly related to the degree of stringency of the hybridization conditions employed. Rawn, J. David, Biochemistry, Pages 993-994 (Carolina Biological Supply Company, 1989); attached as Exhibit B of my Declaration.
12. Persons of ordinary skill in the art of molecular biology know high temperature is an example of a stringent hybridization condition:

Both a temperature too low or an ionic strength too high will reduce the stringency of hybridization and may negatively affect the specificity of the detected signal. In contrast, raising the temperature, decreasing the ionic strength or both, will increase stringency.

Neumaier, M., Braun, A., and Wagener, C. Fundamentals of Quality Assessment of Molecular Amplification Methods in Clinical Diagnostics, Clinical Chemistry, 44:12-26 (1998) (attached as Exhibit C).
13. Persons of ordinary skill in the art of molecular biology know another example of a stringent hybridization condition is the chemical composition of the hybridization solution, such as hybridization solution with relatively low ionic strength (i.e. a relatively low salt concentration). See page 993 of attached Exhibit B. See also Exhibit C (see recited passage in Paragraph 12 above from Exhibit C).
14. Thus, based on the statements of Paragraphs 12 and 13, persons of ordinary skill in the art of molecular biology know the stringency of hybridization conditions may be adjusted by varying the temperature at which hybridization is performed, the chemical composition of the

hybridization solution used during hybridization experiments, or both the hybridization temperature and the chemical composition of hybridization solution.

15. Persons of ordinary skill in the art of molecular biology know time of exposure to hybridization solution at a particular temperature may also be manipulated to attain stringent hybridization conditions; for example, hybridization overnight for a time ranging from twelve to sixteen hours is generally sufficient for most probes and blots to complete base pairing between a nucleic acid sequence and a DNA or mRNA sequence:

Generally, hybridizations are carried on overnight for 12 to 16 hr. This is sufficient for most probes and blots.

Hybridization with Radioactive Probes: Using DNA Fragments as Probes. Current Protocols in Molecular Biology. Section II 6.3.5. (2000). (attached as Exhibit D).

16. Persons of ordinary skill in the art of molecular biology also know another technique for improving specificity of base pairing entails incorporation of non-specific DNA in the hybridization solution:

B. Prehybridization & Hybridization

Since the nylon membrane likes to bind things, background sites are blocked (bound) with non-specific DNA and protein. Usually, sheared salmon sperm DNA is used in prehybe to block these sites.

Instructions posted at <http://catlserver.tamu.edu/ning/ing626.htm> for Lab Session No. 6, Fall 2003: Course Entitled Gene Expression (ANSC/GENE 626 (edited by. N. Ing 9/2/03)); Nancy H. Ing, Instructor; Texas A & M University; College Station, Texas (attached as Exhibit E). The non-specific DNA is said to prevent the probe from hybridizing nonspecifically - that is with nucleic acid that is not complementary (specific) to the probe nucleic acid:

A solution commonly used during probe hybridizations that involve filters (such as Southern, Northern, or Western blots). The solution contains ficoll, bovine serum albumin, polyvinylpyrrolidone (PVP), and a high concentration of nonspecific DNA so the probe won't hybridize nonspecifically.

See Hyperdictionary @ [www.hyperdictionary.com/dictionary/Denhardt's + solution](http://www.hyperdictionary.com/dictionary/Denhardt's+solution); obtained from the Internet on November 12, 2004 (attached as Exhibit F).

17. Persons of ordinary skill in the art of molecular biology know one example of non-specific DNA that may be used to block hybridization of non-specific DNA with the probe nucleic acid is salmon sperm:

Sheared Salmon Sperm DNA is used as a blocking agent to reduce the background in hybridization experiments.

Information page entitled Eppendorf® Sheared Salmon Sperm DNA located at <http://www.brinkmann.com/product.asp?path=115&ref=136>; obtained from the Internet on November 12, 2004 (attached as Exhibit G).

18. Beyond controlling the hybridization stringency directly, by varying the temperature at which hybridization is performed and/or the chemical composition of the hybridization solution used during hybridization experiments (including use of blocking substances as described in Paragraphs 16-17), those of ordinary skill in the art of molecular biology know hybridization stringency may also be controlled by post-hybridization washing conditions:

You can also control for stringency of hybridization at the next step, which is washing the unbound probe away from the blot. If you wash at high temperature or low salt concentration, you will remove any hybrids that are not perfectly matched.

Instructions posted at <http://www.clarkson.edu/class/by412/word/Northern%20hybridization.doc> (obtained from the Internet on November 12, 2004) for Molecular Biology Lab #17 (Northern Transfer and Hybridization Experiment #2: Labeling Probe and Hybridization) taught Fall, 2004 at Clarkson University in Pottsdam, New York by Craig Woodworth (attached as Exhibit H).

19. The invention of the above-identified application is, in one aspect, directed to nucleic acid molecules (and functional variants thereof) , such as (1) single or double-stranded DNA (and

cDNA and genomic DNA) and (2) RNA (and mRNA), that encode porcine adipocyte polypeptide leptin. (Page 1, lines 14-17; page 2, line 29, through page 4, line 9; page 5, lines 17-28; page 7, lines 3-31; page 8 line 6 though page 9, line 27).

20. According to the present invention, the nucleic acid molecules (and functional variants thereof) encoding for porcine leptin polypeptide mentioned in Paragraph 19 hybridize and are capable of hybridizing to nucleotide sequences (and portions thereof), such as various nucleotide sequences (and portions thereof) disclosed in the above-identified application. (Page 8, lines 7-26; page 9, lines 18-27; page 14, line 23 to page 15, line 11).
21. In another aspect, the invention of the above-identified application is directed to methods of hybridizing the nucleic acid molecules encoding for porcine leptin polypeptide (and functional variants thereof) mentioned in Paragraph 19 to nucleotide sequences (and portions thereof), such as various nucleotide sequences (and portions thereof) disclosed in the above-identified application. (page 5, lines 17-28; page 7, lines 3-31; page 8 line 6 though page 9, line 27; page 18, line 27, to page 20, line 8).
22. Example II of the above-identified application provides some particular guidance about hybridization conditions that may be employed when practicing the hybridization methods of the present invention that are the subject of Paragraph 21. This guidance illustrates that stringent hybridization conditions are suitable for practicing the hybridization methods of the present invention.
23. Example II of the above-identified application reads as follows:

ISOLATION OF mRNA CORRESPONDING TO PORCINE LEPTIN cDNA

The porcine leptin cDNA was used as a probe for detection of the full length mRNA. A northern blot containing porcine adipose and bovine adipose poly A+mRNA as well as ob/ob mouse adipose total RNA was provided by Dr. M.

Spurlock of Purina Mills Inc. The blot was hybridized with a [³²P] dCTP labeled porcine leptin cDNA in hybridization solution (HY; 0.9 M NaCl, 0.09 M sodium citrate, 0.05% ficoll, 0.05% polyvinylpyrrolidone, 0.05% BSA, 0.5% SDS, 0.1% sodium pyrophosphate, 10 mM EDTA and 100 mg/ml sonicated salmon sperm DNA at 60°C for 15 hours. The blot was washed to a final stringency of 0.2xSSC (0.03 M NaCl, 0.003 M sodium citrate), 0.1% SDS at 60°C and exposed to X-ray film. A 3,090 bp leptin mRNA was detected in porcine and bovine adipose tissue and a 3,240 bp leptin mRNA was detected on ob/ob mouse adipose tissue. As shown in FIG. 5, lanes 1 and 2 contain the porcine adipose poly A+mRNA, lane 3 contains the adipose total RNA from a control mouse and lanes 4 and 5 contain the adipose total RNA from an ob/ob mouse, and lane 6 contains the bovine adipose poly A+mRNA.

24. Thus, Example II discloses a hybridization technique using porcine leptin cDNA as a probe to detect full length porcine leptin mRNA.
25. According to Example II, porcine leptin cDNA was hybridized against porcine leptin mRNA at 60°C for fifteen hours.
26. According to WO 02/036829A2, a hybridization temperature of about 60°C is generally sufficient to establish stringent conditions for even long probes:

Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes or primers (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes or primers (e.g., greater than 50 nucleotides).

Page 12, lines 2-6. (Attached as Exhibit I).

27. Also, an industry source indicates use of a temperature lower than 60°C (specifically, 50°C in numbered para. 4 on the first page of Exhibit K, 55°C in numbered para. 4 on the second page of Exhibit K, and 50°C in numbered para. 4 on the third page of Exhibit K) is adequate to produce stringent conditions using a non-formamide hybridization solution. Connolly, Amy

L. and Jones, Teri L., Hybridization Buffers I: Comparison of Formamide vs. Aqueous Hybridization Solutions, four pages (KPL Research & Development August, 2002) (attached as Exhibit K). See page 13, lines 6-7, of WO 02/036829A2 of Exhibit I that reference use of formamide to enhance hybridization stringency.

28. The evidence provided in Paragraphs 25-27 regarding use of a hybridization temperature of 60°C during the hybridization trial of Example II of the present application illustrates, to those of ordinary skill in the art of molecular biology, use of stringent hybridization conditions during the hybridization trial of Example II. This statement is probative as to stringency even if the salt concentration in the hybridization solution of Example II is not taken into account. (See Paragraph 14 above).
29. Next, we consider the hybridization time of fifteen hours employed in Example II. See Paragraphs 23 and 25 above.
30. Hybridization overnight for a time ranging from twelve to sixteen hours is generally sufficient for most probes and blots to complete base pairing between a nucleic acid sequence and a DNA or mRNA sequence:

Generally, hybridizations are carried on overnight for 12 to 16 hr. This is sufficient for most probes and blots.

See page 3 of Exhibit D
31. Others in the molecular biology industry indicate hybridization overnight (specifically, numbered para. 4 on the first page of Exhibit K, numbered para. 4 on the second page of Exhibit K, and numbered para. 4 on the third page of Exhibit K) is adequate to produce stringent conditions using a non-formamide hybridization solution. See Exhibit K. See Paragraph 27 above.

32. The evidence provided in Paragraphs 29-31 regarding hybridization for fifteen hours, which clearly qualifies as overnight, during the hybridization trial of Example II of the present application illustrates, to those of ordinary skill in the art of molecular biology, use of stringent hybridization conditions during the hybridization trial of Example II.
33. Next, we consider the salt concentration employed in the hybridization solution of Example II.. Specifically, since the hybridization solution contained 0.9 M NaCl and 0.09 M sodium citrate, one of ordinary skill in the art of molecular biology would understand the hybridization solution contained 0.99 M sodium ion. See Paragraph 23 above.
34. According to WO 02/036829A2, salt concentrations of 0.99 M sodium ion are considered to be low salt concentration generally sufficient to establish stringent conditions for even long probes:
- Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes or primers (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes or primers (e.g., greater than 50 nucleotides).
- Page 12, lines 2-6 of Exhibit I.
35. The evidence provided in Paragraphs 33-34 regarding use of a hybridization solution with a salt concentration of 0.99 M sodium ion during the hybridization trial of Example II of the present application illustrates, to those of ordinary skill in the art of molecular biology, use of stringent hybridization conditions during the hybridization trial of Example II. This statement is probative as to stringency even if the hybridization temperature of Example II is not taken into account. (See Paragraph 14 above).
36. Next, we consider the salmon sperm concentration of 100 mg/ml that is employed in the hybridization solution of Example II. See Paragraph 23 above.

37. Persons of ordinary skill in the art of molecular biology also know another technique for improving specificity of base pairing entails incorporation of non-specific DNA in the hybridization solution. See Exhibit E, as discussed in Paragraph 16 above. The non-specific DNA is said to prevent the probe from hybridizing nonspecifically - that is with nucleic acid that is not complementary (specific) to the probe nucleic acid. See Exhibit F, as discussed at Paragraph 16 above.

38. Salmon sperm at a concentration of 100 mg/ml is incorporated by those skilled in the art of molecular biology to complement and support high stringency hybridization by limiting non-specific nucleic acid binding during hybridization:

In a most preferred embodiment, a nucleic acid sequence that is hybridizable to a nucleic acid sequence (or a complement of the foregoing) encoding the aforementioned peptides, or a derivative of the same, under conditions of high stringency is provided. By way of example and not limitation, procedures using such conditions of high stringency are as follows: Step 1: Filters containing DNA are pretreated for 8 hours to overnight at 65°C in buffer composed of 6× SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 μg/ml denatured salmon sperm DNA. Step 2: Filters are hybridized for 48 hours at 65°C in the above prehybridization mixture to which is added 100 mg/ml denatured salmon sperm DNA and 5-20×10⁶ cpm of ³²P-labeled probe.

Column 14, lines 33-47, of US Patent No. 6777388 (attached as Exhibit J).

39. The evidence provided in Paragraphs 36-38 regarding use of a hybridization solution with a salmon sperm concentration of 100 mg/ml during the hybridization trial of Example II of the present application illustrates, to those of ordinary skill in the art of molecular biology, use of conditions that inhibit non-specific probe hybridization and therefore further support attainment of results consistent with use of stringent conditions during the hybridization trial of Example II.

40. We next consider the post-hybridization washing conditions of Example II that entailed washing "to a final stringency of 0.2xSSC (0.03 M NaCl, 0.003 M sodium citrate), 0.1% SDS at 60°C. See Paragraph 23 above.

41. As noted above, beyond controlling the hybridization stringency directly, by varying the temperature at which hybridization is performed and/or the chemical composition of the hybridization solution used during hybridization experiments, those of ordinary skill in the art of molecular biology know hybridization stringency may also be controlled by post-hybridization washing conditions, such as the temperature and/or the salt concentration. See Paragraph 18 above. For example:

You can also control for stringency of hybridization at the next step, which is washing the unbound probe away from the blot. If you wash at high temperature or low salt concentration, you will remove any hybrids that are not perfectly matched.

See Exhibit H.

42. In this regard, those of ordinary skill in the art recognize that high stringency washing may be accomplished using the hybridization temperature in combination with a final washing solution containing 0.2X SSC and 0.1% SDS, as was employed in Example II:

Following hybridisation, high stringency washing may be done in several steps, with a final wash (about 30 min) at the hybridisation temperature in 0.2-0.1 times SSC, 0.1% SDS.

Paragraph 0188 of US20040137492A1 (attached as Exhibit L).

43. The evidence provided in Paragraphs 40-42 demonstrates those of ordinary skill in the art of molecular biology recognize that high stringency washing may be accomplished using a final washing solution containing 0.2X SSC and 0.1% SDS, as was employed in Example II, and also demonstrates this stringent washing solution be employed at the hybridization temperature to further enhance the washing stringency.

44. The facts presented in Paragraphs 22-43, as supplemented by Exhibits B-D and the Exhibits referenced in Paragraphs 22-43 of my Declaration, illustrate that one of ordinary skill in the art of molecular biology, upon reviewing the hybridization conditions employed in Example II of the above-identified application, would recognize these hybridization conditions as being stringent hybridization conditions that would typically enable a probe, such as the porcine leptin cDNA molecule employed in Example II, to hybridize to a perfect or near perfect nucleic acid molecule complement of the probe.
45. Example III of the above-identified application also provides some particular guidance about hybridization conditions that may be employed when practicing the hybridization methods of the present invention that are the subject of Paragraph 21. This guidance further illustrates that stringent hybridization conditions are suitable for practicing the hybridization methods of the present invention.
46. Example III of the above-identified application reads as follows:

ISOLATION OF GENOMIC DNA CLONE CORRESPONDING TO PORCINE LEPTIN

The porcine leptin cDNA was also used to screen a porcine genomic DNA library. Specifically, a porcine genomic library containing 4.64×10^5 recombinants was previously constructed in SuperCos 1 (Stratagene, Inc.) and screened for porcine leptin. Specifically, two sets of replica filters were prehybridized for 2 h at 60°C. Filters were hybridized overnight with [32 P] dCTP labeled probe at 5×10^5 cpm per ml of hybridization solution at 65°C. Filters were sequentially washed in 2xSSC (0.3 M NaCl, 0.03 M sodium citrate), 0.5% SDS; 1xSSC, 0.5% SDS; and 0.2xSSC, 0.5% SDS with each wash at 60°C for 30 min. Positive clones that showed signals on both replica filters were recovered from the agar plates and individual colonies were isolated by a second low density replica plating and hybridization step. A cosmid designated Obg-361 was isolated that hybridized to the porcine ob cDNA probe and had essentially the same restriction enzyme digestion pattern as found in porcine genomic DNA.

FIG. 6 illustrates the isolation of the cosmid Obg-361. Specifically, lanes 1-4 are an agarose gel containing Kb ladder molecular mass markers (lane 1), cosmid Obg-361 digested with Eco RI (lane 2) and Hind III (lane 3) and biotinylated lambda/Hind III molecular mass markers (lane 4).

Southern blot analysis of the gel in lanes 2-4 were probed with the porcine leptin cDNA indicate that the EcoRI fragments (lane 5) and the Hind III fragments (lane 6) contain leptin sequences. Lane 7 is lambda/Hind III molecular mass markers.

Porcine genomic DNA digested with BAM HI (lane 8), EcoRI (lane 9) and Hind III (lane 10) and hybridized with a Bsa I fragment (300 bp) of the porcine leptin cDNA showed equivalent bands that contain leptin sequences indicating that the porcine leptin gene was isolated in cosmid Obg-361.

The 5917 bp Hind III fragment was subcloned into Bluescript II SK+ (Stratagene, Inc.). Both strands of the sequence was determined using progressive nested deletions using Exonuclease III and Mung Bean nuclease. Sequencing reactions were carried out with Sequenase V2.0. This sequence was 5917 bp in length and contains the entire coding region in two exons (FIGS. 1A-1D (SEQ ID NO:1). There was 78.6% nucleotide identity between the pig and human as well as 71.2% nucleotide identity between pig and mouse coding sequences. The splice junctions for the two exons were confirmed by the cDNA sequence. The cDNA sequence of the protein coding region is shown in FIG. 2 (SEQ ID NO:1 and SEQ ID NO:2). The 501 bp sequences encodes 166 amino acid residue leptin polypeptide with a predicted molecular mass of 18,334 Da.

A clone was obtained using the process described above, Obg H3-15, was deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md., 20852-1776, on Jul. 11, 1996, and has been designated ATCC No. 97653. This microorganism was deposited under the conditions of the Budapest Treaty on the International Recognition of Deposit of Microorganisms for the purpose of Patent Procedure. All restrictions on the availability to the public of the material so deposited will be irrevocably removed upon the granting of a patent. This deposit will be maintained for a time period of 30 years from the date of deposit or 5 years after the last request for the material, whichever is longer.

47. Thus, Example III discloses a hybridization trial that used porcine leptin cDNA as a probe to screen a porcine genomic DNA library and detect porcine leptin DNA.
48. According to Example III, the porcine leptin cDNA was hybridized against the porcine leptin DNA at 65°C overnight.
49. Those skilled in the art of molecular biology recognize that a hybridization temperature of about 60°C is generally sufficient to establish stringent conditions for even long probes:
- Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes or primers (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes or primers (e.g., greater than 50 nucleotides).
- Page 12, lines 2-6, of Exhibit I.
50. Also, an industry source indicates use of a temperature lower than 60°C (specifically, 50°C in numbered para. 4 on the first page of Exhibit K, 55°C in numbered para. 4 on the second page of Exhibit K, and 50°C in numbered para. 4 on the third page of Exhibit K) is adequate to produce stringent conditions using a non-formamide hybridization solution. See page 13, lines 6-7, of Exhibit I.
51. The evidence provided in Paragraphs 48-52 regarding stringent hybridization temperatures of 60°C or less illustrates, to those of ordinary skill in the art of molecular biology, that use of a hybridization temperature of 65°C, as in Example III of the present application, qualifies as a stringent hybridization condition. This statement is probative as to stringency even if the salt concentration in the hybridization solution of Example III is not taken into account. (See Paragraph 14 above).

52. Next, we consider the overnight hybridization time employed in Example III. See Paragraph 46 above.

53. Hybridization overnight is generally recognized by those of ordinary skill in the art of molecular biology as being sufficient for most probes and blots to complete base pairing between a nucleic acid sequence and a DNA sequence:

Generally, hybridizations are carried on overnight for 12 to 16 hr. This is sufficient for most probes and blots.

See page 3 of Exhibit D.

54. Others in the molecular biology industry indicate hybridization overnight (specifically, numbered para. 4 on the first page of Exhibit K, numbered para. 4 on the second page of Exhibit K, and numbered para. 4 on the third page of Exhibit K) is adequate to produce stringent conditions using a non-formamide hybridization solution.

55. The evidence provided in Paragraphs 52-54 regarding hybridization overnight during the hybridization trial of Example III of the present application illustrates, to those of ordinary skill in the art of molecular biology, use of stringent hybridization conditions during the hybridization trial of Example III.

56. We next consider the post-hybridization washing conditions of Example III that concluded with a final wash containing "0.2x SSC, 0.5% SDS . . . at 60°C for 30 min." See Paragraph 46 above.

57. As noted above, beyond controlling the hybridization stringency directly, by varying the temperature at which hybridization is performed and/or the chemical composition of the hybridization solution used during hybridization experiments, those of ordinary skill in the

art of molecular biology know hybridization stringency may also be controlled by post-hybridization washing conditions, such as the temperature and/or the salt concentration. See Paragraph 18 above. For example:

You can also control for stringency of hybridization at the next step, which is washing the unbound probe away from the blot. If you wash at high temperature or low salt concentration, you will remove any hybrids that are not perfectly matched.

See Exhibit H.

58. In this regard, those of ordinary skill in the art recognize that high stringency washing may be accomplished using the hybridization temperature in combination with the final washing solution of Example III that contained 0.2X SSC and 0.1% SDS:

Filters were washed under stringent conditions (0.2x SSC, 0.5% SDS at 60°C).

See page 1076 of Selmi-Ruby, Samia; Watrin, Chantal; Trouttet-Masson, Severine; Bernier-Valentin, Françoise; Flachon, Virginie; Munari-Silem, Yvonne; and Rousset, Bernard; The Porcine Sodium/Iodide Symporter Gene Exhibits an Uncommon Expression Pattern Related to the Use of Alternative Splice Sites not Present in the Human or Murine Species; Endocrinology Vol. 144, No. 3, pages 1074-1085 (2003) (attached as Exhibit M).

59. The evidence provided in Paragraphs 56-58 demonstrates those of ordinary skill in the art of molecular biology recognize that high stringency washing may be accomplished using a final washing solution containing 0.2x SSC and 0.5% SDS at 60°C, as was employed in Example III.
60. The facts presented in Paragraphs 45-59, as supplemented by Exhibits B-D and the Exhibits referenced in Paragraphs 45-59 of my Declaration, illustrate that one of ordinary skill in the art, upon reviewing the hybridization conditions employed in Example III of the above-

identified application, would recognize these hybridization conditions as being stringent hybridization conditions that would typically enable a probe, such as the porcine leptin cDNA molecule employed in Example III, to hybridize to a perfect or near perfect nucleic acid molecule complement of the probe, such as porcine leptin DNA.

61. As noted herein, the invention of the above-identified application is, in one aspect, directed to a variety of nucleic acid molecules (and functional variants thereof) that encode porcine adipocyte polypeptide leptin and are capable of hybridizing to nucleotide sequences (and portions thereof), and in another aspect is directed to hybridizing the nucleic acid molecules (and functional variants thereof) to nucleotide sequences (and portions thereof), such as various nucleotide sequences (and portions thereof) disclosed in the above-identified application. See Paragraphs 19-21 above.
62. The present application discloses conditions for hybridizing the nucleic acid molecules addressed in the present application (and in Paragraph 61) primarily in Examples II and III. As explained in Paragraphs 22, 44-45, and 60, one of ordinary skill in the art, upon reviewing the hybridization conditions employed in Examples II and III of the above-identified application, would recognize these hybridization conditions as being stringent hybridization conditions that would typically enable a probe, such as the porcine leptin cDNA molecule employed in Examples II and III, to hybridize to a perfect or near perfect nucleic acid molecule complement of the probe, such as porcine leptin mRNA or porcine leptin DNA.
63. Consequently, since the above-identified application primarily discloses to one of ordinary skill in the art of molecular biology use of stringent hybridization conditions for hybridizing the nucleic acid molecules addressed in the present application (and in Paragraph 61), it is evident that use of stringent hybridization conditions for hybridizing the nucleic acid

molecules addressed in the present application is in fact disclosed and described in the present application.

64. We next consider the Examiner's comments in support of the Examiner's contention that U.S. Patent No. 6,309,853 to Friedman (subsequently referred to as "the Friedman patent") allegedly renders obvious certain aspects of the invention of the above-identified application directed to nucleic acids which encode porcine leptin and are capable of hybridizing to certain porcine leptin sequences (such as DNA and mRNA):

The instant claims are directed to isolated nucleic acids which encode porcine leptin and hybridize SEQ ID NO:3 or a "functional derivative thereof" (see claims 22, 27) or "variant" (see claims 24-26). The prior art of Friedman et al. (U.S. Pat. No. 6,309,853) disclose nucleic acids which encode human and mouse leptin, which would be considered functional derivatives and/or variants of SEQ ID NO:3 since they encode leptin molecules and would possess similar functional properties as those of the porcine leptin, absent evidence to the contrary. Friedman et al. teach that the leptin gene (or OB) could be isolated from domestic animals using the methods disclosed therein (see column 26, line 53 to column 27, line 49). Friedman et al. specifically mention swine as a domestic animal for which leptin would be useful (see column 48, lines 41-47). Friedman et al. do not specifically disclose an isolated nucleic acid encoding a porcine leptin polypeptide. However, it would have been obvious to use the nucleic acid of Friedman et al. encoding human or mouse leptin and hybridize it to a porcine DNA library and isolate a nucleic acid molecule encoding porcine leptin because Friedman et al. teach methods for isolating leptin encoding nucleic acids and also teach that it would be beneficial to administer leptin to swine. Therefore, the invention as a whole would have been obvious at the time it was made, absent evidence to the contrary.

Office Action dated June 3, 2004 in the above-identified application.

65. I am experienced in isolating, purifying, and manipulating DNA sequences, such as porcine, bovine, murine, and human DNA sequences, such as leptin DNA sequences, due to my

education and work experience relating to DNA sequence and leptin genetics issues over the past twenty-five years. See Paragraphs 1-8 above.

66. I am familiar with and have carefully and thoroughly reviewed the Friedman patent which the Examiner relies upon in the Examiner's statement recited in Paragraph 65.
67. Based on my thorough review of the Friedman patent, I note, consistent with the Examiner's observation recited in Paragraph 64, the Friedman patent discloses murine and human leptin DNA sequences and polypeptides.
68. Based on my careful review of the Friedman patent and consistent with the Examiner's observation recited in Paragraph 64, the Friedman patent does not disclose any porcine leptin DNA (or mRNA) molecules or polypeptides.
69. Based on my thorough review of the Friedman patent and consistent with the Examiner's observation recited in Paragraph 64, the Friedman patent does not disclose any functional derivative or variant DNA (or mRNA) molecules that encode for porcine leptin polypeptide.
70. Based on my careful review and comparison of the human and murine leptin DNA sequences disclosed in the Friedman patent and the porcine leptin DNA sequences disclosed in the above-identified application, it is my observation that the human and murine leptin DNA sequences disclosed in the Friedman patent differ substantially from the porcine leptin DNA sequences disclosed in the above-identified application; consequently, the porcine leptin of the present application that is based on the porcine leptin DNA sequences (and functional variants thereof) disclosed in the above-identified application differs in substantial detail from both the human leptin and the murine leptin disclosed in the Friedman patent.

71. As a result, based on my observation in Paragraph 70, the porcine leptin of the present application is functionally, different from both the human leptin and the murine leptin disclosed in the Friedman patent, as discussed more fully in subsequent Paragraphs of my Declaration.
72. For example, when recombinant porcine leptin protein is administered by intracerebroventricular (ICV) injection to crossbred prepuberal gilts (normal female pigs), the crossbred prepuberal gilts exhibit increased growth hormone secretion after the leptin administration. C.R. Barb, X. Yan, M.J. Azain, R.R. Kraeling, G.B. Rampacek, and T.G. Ramsay, Recombinant Porcine Leptin Reduces Feed Intake and Stimulates Growth Hormone Secretion in Swine, Domestic Animal Endocrinology, Vol. 15, No. 1, pages 77-86 (1998) (attached as Exhibit N).
73. On the other hand, when recombinant human leptin protein is administered by ICV injection to normal male rats, the normal male rats do not exhibit increased growth hormone secretion after the leptin administration. E. Carro, R. Senaris, R.V. Considine, F.F. Casanueva, C. Dieguez, Regulation of In Vivo Growth Hormone Secretion by Leptin, Endocrinology, Vol. 138, No. 5, pages 2203-2206 (1997) (attached as Exhibit O).
74. Since recombinant porcine leptin protein administration increases growth hormone secretion in pigs, while recombinant human leptin protein administration fails to increase growth hormone secretion in male rats, the effects of porcine leptin protein administration and human leptin protein administration differ dramatically, and it is consequently evident the porcine leptin protein is functionally very different from the human leptin protein.
75. I have carefully reviewed and am familiar with the publication of Exhibit N by C.R. Barb, X. Yan, M.J. Azain, R.R. Kraeling, G.B. Rampacek, and T.G. Ramsay, Recombinant Porcine

Leptin Reduces Feed Intake and Stimulates Growth Hormone Secretion in Swine, Domestic Animal Endocrinology, Vol. 15, No. 1, pages 77-86 (1998) (subsequently referred to as “the Barb publication”) that documents the increased growth hormone secretion by pigs after recombinant porcine leptin protein administration.

76. In mammals, leptin receptors are expressed in the hypothalamus, pituitary, adipose tissue, ovary and some other additional organs. The two primary regulators of growth hormone secretion in the pig brain, namely growth-hormone releasing factors and somatostatin, are produced in the arcuate and ventromedial hypothalamus of the pig brain. (See the Introduction section on page 77 of the Barb publication of Exhibit N).
77. The arcuate and ventromedial hypothalamus of the pig brain are involved in food intake regulation. Therefore, the authors of the Barb publication conducted several experiments to test the hypothesis that leptin modulates feed intake, growth hormone, insulin-like growth factor, insulin and thyroxine secretion in the pig. (See the Introduction section on pages 77-78 of the Barb publication of Exhibit N).
78. According to the Barb publication, two experiments (EXP I and EXP II) were conducted to test the hypothesis that porcine leptin affects growth hormone, insulin-like growth factor-1 (IGF-1), insulin, thyroxine (T_4) secretion, and feed intake. (See the Introduction section on page 78 of the Barb publication of Exhibit N).
79. In a first experiment (identified as EXP I), eight crossbred prepuberal gilts (normal female pigs) that weighed about 80.6 ± 2.7 kg each and were about 150 days old were first surgically implanted surgically with lateral intracerebroventricular (ICV) cannulas using a steriotaxic procedure. (See the EXP I section on page 78 of the Barb publication of Exhibit N).

80. The female pigs were then individually penned in an environmentally controlled building and exposed to both a constant temperature of 22°C and artificial 12-hr:12-hr light:dark illumination periods. Each day, the female pigs were fed a corn-soybean meal ration containing 14% crude protein and supplemented with vitamins and minerals in accordance with the National Research Council guidelines at 0800 hour and at 1700 hour. (See the EXP I section on page 78 of the Barb publication of Exhibit N).
81. All of the female pigs were fitted with an indwelling jugular vein cannulae twenty-four hours before administration of the recombinant porcine leptin protein. On the day the recombinant porcine leptin protein was to be administered, the female pigs were fed at 0800 hour, and blood sampling started at 0900 hour. (See the EXP I section on page 78 of the Barb publication of Exhibit N).
82. Blood samples were collected starting at 0900 hour from each female pig every fifteen minutes for four hours before and three hours after ICV injections of 150 µl 0.9% saline, or 10 µg, 50 µg, or 100 µg of recombinant porcine leptin protein in 150 µl of 0.9% saline. (See the EXP I section on page 78 of the Barb publication of Exhibit N).
83. The recombinant porcine leptin protein was derived from the porcine leptin cDNA sequence (best depicted by the sequence identified as SEQ ID NO:4 of the above-identified application) representing the secreted porcine leptin protein (amino acids 22 to 167) (best depicted by the sequence identified as SEQ ID NO:5 of the above-identified application). (See the EXP I section on page 78 of the Barb publication of Exhibit N). The following steps were used to prepare purified recombinant porcine leptin protein that was administered to the female pigs:

- A. The porcine leptin cDNA was amplified by polymerase chain reaction (PCR) from porcine leptin cDNA containing plasmid (PCR2.1) (Invitrogen, Carlsbad, CA) that harbored the complete coding region of the porcine leptin cDNA.
- B. The porcine leptin cDNA was subcloned in-frame into a procaryotic expression vector pGEX-2T (Pharmacia Biotech, Piscataway, NJ) and confirmed by DNA sequencing.
- C. The cDNA construct present in the procaryotic expression vector pGEX-2T was transformed and over-expressed in *E. coli* (JM109). A crude porcine leptin protein extract containing the GST-leptin fusion was prepared by conducting sonification and precipitation.
- D. After precipitation, the crude recombinant porcine leptin protein was filtered and subsequently loaded onto a prepacked glutathione sepharose 4B column and was washed with 30-bed volumes of PBS.
- E. The column was then incubated with thrombin solution. The recombinant porcine leptin protein was finally recovered in the flow through and the subsequent washes.
- F. Refolding of the recombinant porcine leptin protein was achieved by denaturing the protein in 4 M urea and dialyzing the denatured protein successively against 3 M, 2 M, 1 M and 0.5 M urea and three changes of phosphate buffer solution (PBS). Each dialysis step was performed for twelve hours in 10,000 MW cutoff tubing at 4°C against fifty volumes of PBS solution.

G. Purity of the recombinant porcine leptin protein was estimated by staining a SDS-PAGE gel with coomassie blue; recombinant porcine leptin protein was the only band detectable on the gel. The purified recombinant porcine leptin protein was subsequently used in EXP I and EXP II.

(See the EXP I section on page 78 of the Barb publication of Exhibit N).

84. According to the Barb publication, serum growth hormone concentrations among the female pigs were similar before conducting EXP I. However, serum growth hormone concentrations increased after injection of 10 µg, 50 µg, and 100 µg of recombinant porcine leptin protein to the female pigs when compared to the serum growth hormone concentrations of the saline-treated (control) female pigs. (See the Results section for EXP I on page 80 of the Barb publication of Exhibit N).
85. This increased serum growth hormone concentration mentioned in Paragraph 84 after recombinant porcine leptin protein administration in normal female pigs is in contrast to the effects observed after recombinant human leptin protein administration to normal male rats, where recombinant human leptin protein administration did not increase growth hormone secretion in normal male rats. See Paragraphs 72 and 73 above.
86. I have carefully reviewed and am familiar with the publication of Exhibit O by E. Carro, R. Senaris, R.V. Considine, F.F. Casanueva, C. Dieguez, Regulation of In Vivo Growth Hormone Secretion by Leptin, Endocrinology, Vol. 138, No. 5, pages 2203-2206 (1997) (subsequently referred to as "the Carro publication") that documents the failure of growth hormone secretion by normal male rats to increase after recombinant human leptin protein administration.

87. According to the Carro publication, adult male Sprague-Dawley rats weighing from 200 grams to 250 grams each were housed in a temperature and humidity controlled room and exposed to 12-hour:12-hour light:dark illumination periods in a manner similar to the environmental conditions reported in the Barb publication. (See the Animals and Experimental Procedure section on page 2203 of the Carro publication of Exhibit O).
88. Also, chronic intracerebroventricular (ICV) and intracardiac canulae similar to those of the Barb publication, were implanted in the normal male rats using sodium pentobarbital anesthesia. (See the Animals and Experimental Procedure section on page 2203 of the Carro publication of Exhibit O).
89. After implantation, the normal male rats implanted per the procedure mentioned in Paragraph 88 were placed into isolation test chambers for five days and given free access to regular Purina rat chow and tap water. (See the Animals and Experimental Procedure section on page 2203 of the Carro publication of Exhibit O).
90. Thereafter, the male rats were divided into two groups. One group of the rats (hereinafter referred to as the "fed rats group") continued to have food available ad libitum. A second group of the rats (hereinafter referred to as the "fast rats group") were deprived of food for forty-eight hours before blood sampling. (See the Animals and Experimental Procedure section on page 2203 of the Carro publication of Exhibit O).
91. On the day that recombinant human leptin protein was to be administered to the normal rats, blood samples were withdrawn every fifteen minutes for a period of six hours (10:00 to 16:00) from each rat of both the fed rats group and the fast rats group. (See the Animals and Experimental Procedure section on page 2203 of the Carro publication of Exhibit O).

92. At 10:15 of the treatment day, fifteen minutes after taking the first blood sample, the fed rats group and the fast group rats were each further subdivided into four treatment subgroups; each rat of the four treatment subgroups of the fed rats group and each rat of the four treatment subgroups of the fast rats group received an ICV injection of either (1) 10 μ l normal rabbit serum, (2) 10 μ l of specific antiserum against leptin, (3) 10 μ l of vehicle, or (4) 10 μ l containing 10 μ g of recombinant human leptin protein. (See the Animals and Experimental Procedure section on pages 2203-2204 of the Carro publication of Exhibit O).
93. The amount of recombinant human leptin protein (10 μ g) administered to the normal male rats during the study documented in the Carro publication and the amount of recombinant porcine leptin protein (10 μ g) administered to some of the normal female pigs during the study documented in the Barb publication was the same. See Paragraphs 84 and 92.
94. According to the Carro publication, ICV administration of recombinant human leptin protein to the rats of the fed rats group did not modify the pulsatile secretion of growth hormone apart from a slight decrease in mean nadir growth hormone levels. (See the Results section extending from page 2204 to page 2205 of the Carro publication of Exhibit O).
95. Therefore, the authors of the Carro publication concluded that leptin administration to normal fed males rats fails to increase growth hormone secretion:
While leptin administration to normal fed rats did not modify spontaneous GH secretion
(See the Abstract section on page 2205 of the Carro publication of Exhibit O).
96. This observed non-effect by the authors of the Carro publication contrasts sharply to the observed increase in growth hormone secretion after ICV administration of recombinant porcine leptin protein to normal fed female pigs, as detailed in the Barb publication. See Paragraphs 94-95 versus Paragraph 84.

97. Therefore, based on the documented differences in growth hormone secretion after ICV administration to normal fed pigs (per the Barb publication) versus normal fed rats (per the Carro publication), it is evident that recombinant porcine leptin protein surprisingly functions very differently from recombinant human leptin protein upon administration to mammals. See Paragraphs 73-74 and 96.
98. As another example, when recombinant porcine leptin is administered by intracerebroventricular (ICV) injection to crossbred prepuberal gilts (normal female pigs), the recombinant porcine leptin administration fails to change thyroxine (T_4) secretion. (See the Results section for EXP I on pages 80-81 of the Barb publication of Exhibit N).
99. On the other hand, when recombinant mouse leptin is administered by ICV injection to normal ad libitum fed male rats, the normal ad libitum fed male rats exhibit significantly decreased thyroxine (T_4) levels in the blood after the recombinant mouse leptin administration. Isabelle Cusin, Juha Rouru, Theo Visser, Albert G. Burger, and Francoise Rohner-Jeanrenaud, Involvement of Thyroid Hormones in the Effect of Intracerebroventricular Leptin Infusion on Uncoupling Protein-3 Expression Rat Muscle, Diabetes, Vol. 49, pages 1101-1105 (2000) (attached as Exhibit P).
100. Since recombinant porcine leptin administration fails to change thyroxine (T_4) secretion in pigs, while recombinant murine leptin administration significantly decreased thyroxine (T_4) levels in the blood of male rats, the effects of porcine leptin administration and murine leptin administration differ dramatically, and it is evident the porcine leptin functions very different from the murine leptin.
101. I have carefully reviewed and am familiar with the publication of Exhibit P by Isabelle Cusin, Juha Rouru, Theo Visser, Albert G. Burger, and Francoise Rohner-Jeanrenaud,

Involvement of Thyroid Hormones in the Effect of Intracerebroventricular Leptin Infusion on Uncoupling Protein-3 Expression Rat Muscle, Diabetes, Vol. 49, pages 1101-1105 (2000) (subsequently referred to as “the Cusin publication”) that documents the significantly decreased thyroxine (T_4) levels in the blood of the male rats after the leptin administration.

102. In the study documented in the Cusin publication, normal male rats (eight to nine week old male Sprague-Dawley rats) were housed in individual cages under conditions of controlled temperature (23 °C) and a 12 hour:12 hour light:dark illumination period in a manner similar to the environmental conditions in a manner similar to the environmental conditions reported in the Barb publication and in the Carro publication. The rats were allowed ad libitum access to water and standard laboratory diet before initiation of the study. (See the Research Design and Methods section on pages 1101-1102 of the Cusin publication of Exhibit P).

103. The normal male rats were divided into three groups:

- Group 1: Treated rats ICV infused with recombinant mouse leptin protein in leptin vehicle (0.1 mol/L Tris buffer (pH 9) for the leptin;
- Group 2: Control rats fed ad libitum and ICV infused with leptin vehicle (0.1 mol/L Tris buffer (pH 9)) only without any leptin;
- Group 3: Control rats ICV infused with leptin vehicle (0.1 mol/L Tris buffer (pH 9)) only without any leptin, but pair-fed to the amount of food consumed by the Group 1 (leptin-infused) rats.

(See the Research Design and Methods section on p. 1102 of the Cusin publication of Exhibit P).

104. All of the rats of Groups 1-3 were anesthetized and equipped with a cannula positioned in the right lateral ventricle to permit ICV infusion. Osmotic mini-pumps either delivered (1) 12.5 µg of recombinant murine leptin protein in 0.1 mol/L Tris buffer (pH of 9) per day for six days to Group 1 rats or (2) 0.1 mol/L Tris buffer (pH 9) through an ICV infusion cannula

to the Group 2 and Group 3 rats. (See the Research Design and Methods section on page 1102 of the Cusin publication of Exhibit P).

105. According to the Cusin publication, the Group 1 rats (ICV-infused leptin) showed a significant decrease (-28%) in plasma thyroxine (T_4) levels relative to the Group 2 (ad libitum fed control rats). (See the Results section on page 1102, column 2, of the Cusin publication of Exhibit P).
106. The decreased plasma thyroxine (T_4) levels exhibited when recombinant murine leptin protein is administered to normal male rats, as reported in the Cusin publication, stands in stark contrast to the failure of recombinant porcine leptin protein to alter thyroxine (T_4) secretion in normal female pigs, as reported in the Barb publication. Paragraph 105 versus Paragraph 98.
107. Therefore, based on the documented differences in thyroxine (T_4) secretion after ICV administration to normal fed pigs (per the Barb publication) versus thyroxine (T_4) secretion of normal fed rats (per the Cusin publication), it is evident that recombinant porcine leptin protein surprisingly functions very differently from recombinant murine leptin protein upon administration to mammals. See Paragraphs 100 and 106.
108. The foregoing factual evidence illustrates the human leptin disclosed in the Friedman patent does not, despite the Examiner's contentions to the contrary (see Paragraph 64), necessarily, or actually, possess functional properties that are similar to the functional properties of the porcine leptin disclosed in the above-identified application.
109. For example, based on the documented differences in growth hormone secretion after ICV administration to normal fed pigs (per the Barb publication) versus normal fed rats (per the

Carro publication), it is evident that recombinant porcine leptin protein surprisingly functions very differently from recombinant human leptin protein upon administration to mammals. See Paragraphs 72-74 and 96.

110. The documented differences in growth hormone secretion after ICV administration to normal fed pigs (per the Barb publication) versus normal fed rats (per the Carro publication) are surprising and unexpected, since there is no evidence of record, such as in either the Barb publication or the Carro publication, that would suggest the differential effects on growth hormone secretion caused by porcine leptin protein administration in pigs versus human leptin protein administration in rats.
111. Furthermore, the documented differences in growth hormone secretion after ICV administration to normal fed pigs (per the Barb publication) versus normal fed rats (per the Carro publication) are surprising and unexpected, since there is no evidence of record, such as in either the Barb publication or the Carro publication, that would suggest human leptin protein administration in rats would have no effect on growth hormone secretion by the rats, while porcine leptin protein administration in pigs would cause an increase in growth hormone secretion by the pigs.
112. Therefore, based on the factual results noted in Paragraphs 106-111 and despite the Examiner's contentions to the contrary (see Paragraph 64), the Examiner's speculative suggested hybridization of the nucleic acid of the Friedman patent that encodes human leptin to a porcine DNA library and subsequent isolation of a nucleic acid molecule encoding porcine leptin is not suggested since the functional characteristics of human leptin disclosed in the Friedman patent would not confirm isolation of a nucleic acid molecule encoding for porcine leptin as claimed in the above-identified application.

113. Likewise, the foregoing factual evidence illustrates the murine leptin disclosed in the Friedman patent does not, despite the Examiner's contentions to the contrary (see Paragraph 64), necessarily, or actually, possess functional properties that are similar to the functional properties of the porcine leptin disclosed in the above-identified application.
114. For example, based on the documented differences in thyroxine (T_4) secretion after ICV administration to normal fed pigs (per the Barb publication) versus normal fed rats (per the Cusin publication), it is evident that recombinant porcine leptin protein surprisingly functions very differently from recombinant murine leptin protein upon administration to mammals. See Paragraphs 98-100 and 106-107.
115. The documented differences in thyroxine (T_4) secretion after ICV administration to normal fed pigs (per the Barb publication) versus normal fed rats (per the Cusin publication) are surprising and unexpected, since there is no evidence of record, such as in either the Barb publication or the Cusin publication, that would suggest the differential effects on thyroxine (T_4) secretion caused by porcine leptin protein administration in pigs versus murine leptin protein administration in rats.
116. Furthermore, the documented differences in thyroxine (T_4) secretion after ICV administration to normal fed pigs (per the Barb publication) versus normal fed rats (per the Cusin publication) are surprising and unexpected, since there is no evidence of record, such as in either the Barb publication or the Cusin publication, that would suggest murine leptin protein administration in rats would reduce thyroxine (T_4) secretion in the rats, while porcine leptin protein administration in pigs would not affect thyroxine (T_4) secretion by the pigs.
117. Finally, despite the Examiner's contentions to the contrary (see Paragraph 64), the Examiner's speculative suggested hybridization of the nucleic acid of the Friedman patent that encodes

Inventor: Michael E. Spurlock

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mouse leptin to a porcine DNA library and subsequent isolation of a nucleic acid molecule encoding porcine leptin is not suggested since the functional characteristics of murine leptin disclosed in the Friedman patent would not confirm isolation of a nucleic acid molecule encoding for porcine leptin as claimed in the above-identified application.

118. I declare that all statements made herein that are of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

NAME (Printed):

Michael E. Spurlock

SIGNATURE:

Michael E. Spurlock

DATE:

Nov. 23, 2004



First Named

Inventor : Michael E. Spurlock

Appln. No. : 09/932,888

Filed : August 20, 2001

Title : Porcine Leptin Protein, Antisense and Antibody

Docket No. : LL31.12-0016

Group Art Unit: 1647

Examiner: C. J. Saoud

EXHIBIT A

of

DECLARATION

submitted under 37 C.F.R. 1.132

RESUME OF MICHAEL E. SPURLOCK, PH.D.



GENERAL INFORMATION

a. Professional Appointments:

- 1) Postdoctoral Research Associate, University of Missouri, 1989-1990.
- 2) Primary Reviewer, U.S. Food & Drug Administration, Center for Veterinary Medicine, 1990-1991.
- 3) Postdoctoral Research Associate, Purdue University, 1991-1993.
- 4) Research Scientist, Purina Mills, Inc., 1993-1994.
- 5) Senior Research Scientist, Purina Mills, Inc., 1994-1996.
- 6) Research Manager, Purina Mills, Inc., 1997-1998.
- 7) Senior Research Manager, Purina Mills, Inc., 1998-1999.
- 8) Adjunct Assistant Professor, Purdue University, 1995-1999.
- 9) Assistant Professor, Purdue University, 1999-2002.
- 10) Associate Professor, Purdue University, 2002-present.

b. Awards & Honors:

- 1) National Pork Producers' Council, Innovative Basic Research Award, 1994.
- 2) National Pork Producers' Council, Innovative Basic Research Award, 1997.
- 3) ESCOP/ACOP Fellow, 2003-04.
- 4) School of Agriculture Research Award, 2004.

c. Memberships in Academic, Professional, and Scholarly Societies:

- 1) American Society of Animal Science
- 2) American Society for Nutritional Sciences
- 3) Endocrine Society
- 4) American Diabetes Association
- 5) Sigma Xi
- 6) Gamma Sigma Delta

A. EXCELLENCE IN RESEARCH

The overall goal of Dr. Spurlock's research is to develop innovative technologies that improve the efficiency of animal growth and support the consumer's desire for highly palatable, yet healthy, meat products. Experimental approaches encompass whole-animal and cell culture experimentation, and range from the collection of growth performance data to detailed evaluations of gene regulation and signal transduction. Two avenues of research are actively pursued. First, his laboratory seeks to define the roles that adipocyte-derived hormones and cytokines play in the regulation of the overall energy balance and partitioning among tissues. Secondly, he is actively involved in delineating the mechanisms by which stress and disease suppress growth and influence the composition of body weight gain. The latter project is currently directed at understanding immunological aspects of the adipocyte with a particular emphasis on the immunological role of adiponectin. Dr. Spurlock is also an active member of the Comparative Medicine Program, and is concentrating on developing the pig as a biomedical model. He has an ongoing collaborative project with Dr. Michael Sturek, Indiana University School of Medicine, to establish the Ossabaw breed of minipig as a model for the human metabolic syndrome. His research in this area focuses on inflammation in the adipocyte and its relationship to insulin sensitivity. Recent publications from his laboratory in this topic have received national and international media attention.

1. Published Work (*indicates primary author(s)):

a. Refereed papers:

- 1) Spurlock, M.E.*, and J.E. Savage*. 1992. Antioxidant activity of Japanese quail liver cytosol in the absence and presence of reduced glutathione. *Poultry Sci.* 71:928-931.
- 2) Spurlock, M.E.*, J.D. Browning*, and B.L. O'Dell*. 1992. Low zinc status in guinea pigs and chicks has no effect on reassembly rate of brain microtubules. *J. Nutr. Biochem.* 3:594-598.
- 3) Spurlock, M.E.*, and J.E. Savage*. 1993. Effects of dietary protein, sulfur amino acids and antioxidants on fatty liver hemorrhagic syndrome induced in Japanese quail by dietary modifications. *Poultry Sci.* 72:2095-2105.
- 4) Spurlock, M.E.*, J.C. Cusumano, and S.E. Mills*. 1993. (-)-[³H]-Dihydroalprenolol binding to β -adrenergic receptors in porcine adipose tissue and skeletal muscle membrane preparations. *J. Anim. Sci.* 71:1778-1785.
- 5) Spurlock, M.E.*, J.C. Cusumano, and S.E. Mills*. 1993. The affinity of ractopamine, clenbuterol and L-644,969 for the β -adrenergic receptor population in porcine adipose tissue and skeletal muscle membrane. *J. Anim. Sci.* 71:2061-2065.
- 6) Spurlock, M.E.*, J.C. Cusumano, S.Q. Ji, C.K. Smith, II, D.B. Anderson*, D.L. Hancock*, and S.E. Mills*. 1994. The effect of ractopamine on β -adrenoceptor density and affinity in porcine adipose and skeletal muscle tissue. *J. Anim. Sci.* 72:75-80.

- 7) McComb, M.A.* and M.E. Spurlock*. 1996. Expression of stress proteins in porcine tissues: Developmental changes and effect of immunological challenge. *J. Anim. Sci.* 75:195-201.
- 8) Spurlock, M.E.*, K.J. Hahn, and J.L. Miner*. 1996. Regulation of adipsin and body composition in the monosodium glutamate (MSG) mouse. *Physiol. Behav.* 60:1217-1221.
- 9) Spurlock, M.E.*, G.R. Frank, G.M. Willis, J.L. Kuske, and S.G. Cornelius. 1996. Effect of dietary energy source and immunological challenge on growth performance and immunological variables in the growing pig. *J. Anim. Sci.* 75:720-726.
- 10) Spurlock, M.E.*. 1997. Regulation of metabolism and growth during immune challenge: An overview of cytokine function. *J. Anim. Sci.* 75:1773-1783.
- 11) Bidwell, C.A.*, S.Q. Ji, G.R. Frank, S.G. Cornelius, G.M. Willis, and M.E. Spurlock*. 1997. Cloning and expression of the porcine obese gene. *Anim. Biotech.* 8:191-206.
- 12) Ji, S.Q.*, R.R. Scott, G.M. Willis, and M.E. Spurlock*. 1998. Partial cloning and expression of the bovine leptin gene. *Anim. Biotech.* 9:1-14.
- 13) Spurlock, M.E.*, G.R. Frank, S.G. Cornelius, S.Q. Ji, G.M. Willis, and C.A. Bidwell*. 1998. *Obese* gene expression in porcine adipose tissue is reduced by food deprivation but not by maintenance or submaintenance intake. *J. Nutr.* 128:677-682.
- 14) Spurlock, M.E.*, M.A. Ranalletta*, S.G. Cornelius, G.R. Frank, S.Q. Ji, G.M. Willis, A.L. Grant*, and C.A. Bidwell*. 1998. Leptin expression in porcine adipose tissue is not increased by endotoxin but is reduced by growth hormone. *J. Int. Cyto. Res.* 18: 1051-1058.
- 15) Ji, S.Q.*, G.R. Frank, S.G. Cornelius, G.M. Willis, and M.E. Spurlock*. 1998. Porcine somatotropin improves growth in finishing pigs without altering Calpain 3 (p94) or α -actin mRNA abundance and has a differential effect on calpastatin transcription products. *J. Anim. Sci.* 76:1389-1395.
- 16) Houseknecht, K.L. *, C.A. Baile*, R.L. Matteri*, and M.E. Spurlock*. 1998. The biology of leptin: A review. *J. Anim. Sci.* 76:1405-1420.
- 17) Ji, S.Q.*, R.L. Losinski*, S.G. Cornelius, G.R. Frank, G.M. Willis, D.E. Gerrard*, F.F.S. Depreux*, and M.E. Spurlock*. 1998. Myostatin expression in porcine tissues: tissue specificity and developmental and postnatal regulation. *Am. J. Physiol.* 275: R1265-R1273.
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- 19) Houseknecht, K.L.*, C.A. Bidwell*, C.P. Portocarrero, and M.E. Spurlock*. 1998. Expression and cDNA cloning of porcine peroxisome proliferator-activated receptor gamma (PPAR γ). *Gene* 225:89-96.
- 20) Ji, S.Q.*, G.M. Willis, G.R. Frank, S.G. Cornelius, and M.E. Spurlock*. 1999. Soybean isoflavones genistein and genistin inhibit myoblast proliferation, fusion, and myotube protein synthesis. *J. Nutr.* 129:1291-1297.
- 21) McCracken, B.A.*, M.E. Spurlock*, M.A. Roos, F.A. Zuckermann, and H.R. Gaskins*. 1999. Weaning anorexia induced local inflammation in the piglet small intestine. *J. Nutr.* 129:613-619.
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- 23) Houseknecht, K.L.*, C.P. Portocarrero, S.Q. Ji, R. Lemenager*, and M.E. Spurlock*. 2000. Growth hormone regulates leptin gene expression in bovine adipose tissue: correlation with adipose IGF-1 expression. *J. Endocrinol.* 164:51-57.
- 24) Leininger, M.T.*, C.P. Portocarrero, A.P. Schinckel, M.E. Spurlock*, C.A. Bidwell, J.N. Nielsen, and K.L. Houseknecht*. 2000. Physiological response to acute endotoxemia in swine: effect of genotype on energy metabolites and leptin. *Dom. Anim. Endocrinol.* 18:71-82.
- 25) Leininger, M.T.*, C.P. Portocarrero, C.A. Bidwell, M.E. Spurlock*, and K.L. Houseknecht*. 2000. Leptin expression is reduced with acute endotoxemia in the pig: correlation with glucose, insulin, and insulin-like growth factor-1 (IGF-1). *J. Int. Cyto. Res.* 20:99-106.
- 26) Spurlock, M.E.*, S.Q. Ji*, J.L. Kuske*, S.G. Cornelius, G.R. Frank, and G.M. Willis. 2001. Changes in the expression of uncoupling proteins and lipases in porcine adipose tissue and skeletal muscle during feed deprivation. *J. Nutr. Biochem.* 12:81-87.
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b. Manuscripts Submitted:

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- 2) Ajuwon, K.M.*, and M.E. Spurlock*. Adiponectin attenuates lipopolysaccharide-induced NFkB activation and IL-6 expression, and up regulates PPAR γ expression in adipocytes. *Am. J. Physiol.*

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- 2) Spurlock, M.E.* and J.E. Savage*. 1989. Enterocyte absorption of glutathione after oral administration and uptake by intestinal brush border vesicles. *Poultry Sci.* 68 (Suppl.1):139.
- 3) Spurlock, M.E.*, J.C. Cusumano, S.Q. Ji, D.B. Anderson, D.L. Hancock*, and S.E. Mills*. 1993. The effect of ractopamine on β -adrenoceptor density and affinity in porcine adipose and skeletal muscle tissue. *J. Anim. Sci.* 71 (Suppl. 1):135.
- 4) Chavis, S.*, D.L. Hancock*, P.J. Ruwe-Kaiser, and M.E. Spurlock*. 1994. The effects of aspirin and *E. coli* lipopolysaccharide on growth, calpain and calpastatin activities in growing pigs. *J. Anim. Sci.* 72 (Suppl. 2):50.
- 5) Miner, J.L.*, K.J. Hahn, M.E. Spurlock*, P.J. Ruwe-Kaiser, and C.A. Baile. 1994. Porcine adipsin; cloning, expression, complement activity, and effect of fasting on serum concentration. *J. Anim. Sci.* 72 (Suppl. 1):74.
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- 7) Mills, S. E.* and M.E. Spurlock*. 1995. Tissue and species variation in the activation of adenylate cyclase by β -adrenergic agonists. *J. Anim. Sci.* 73 (Suppl. 1):145.
- 8) McComb, M.A.*, P.J. Ruwe-Kaiser, and M.E. Spurlock*. 1995. Expression of stress proteins in developing pigs and in growing pigs challenged with endotoxin. *J. Anim. Sci.* 73 (Suppl. 1):64.
- 9) Ji, S.Q., S. Neustrom*, and M.E. Spurlock*. 1996. Effect of tumor necrosis factor alpha on protein metabolism in L8 myotube cultures. *J. Anim. Sci.* 74(Suppl. 1):150.
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- 13) Ji, S.Q.*, G.M. Willis, S.G. Cornelius, and M.E. Spurlock*. 1997. Soybean genistein inhibits myoblast proliferation, differentiation, and myotube protein synthesis. *J. Anim. Sci.* 75 (Suppl. 1):56.
- 14) McComb, M.A.*, J.W. Frank, A.P. Schinckel*, M.E. Spurlock*, B.T. Richert*, P.V. Malven, and A.L. Grant. 1997. Interactive effects of rearing environment, pig genotype, and antibiotic therapy on growth, serum IGF-1, and acute phase proteins. *J. Anim. Sci.* 75 (Suppl. 1):85.
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- 18) Ji, S.Q.*, G.M. Willis, R.R. Scott, and M.E. Spurlock*. 1997. Partial cloning of the bovine leptin gene and its expression in adipose depots and in cattle before and after finishing. *J. Anim. Sci.* 75 (Suppl. 1):167.
- 19) Spurlock, M.E.*, S.G. Cornelius, G.R. Frank*, and G.M. Willis. 1998. Growth performance and immunological variables in pigs fed different fat sources and subjected to multiple immunological challenges. *J. Anim. Sci.* 76 (Suppl. 2):59.
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- 33) N. Mathialagan*, C.J Dyer*, M.T Leininger*, K. Govinderajan, J.C Byatt, F.C Buonomo, and M.E. Spurlock*. 2002. Expression Profiling of low and high lean pigs from weaning to finish. Plant and Anim. Genome Conf. X, San Diego, CA. page 703. http://www.intl-pag.org/pag/10/abstracts/PAGX_703.html.

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- 39) Wulster-Radcliffe*, M.C., J.A. Christian, J. Wang, and M E. Spurlock*. 2004. Adiponectin attenuates the induction of proinflammatory cytokines in pig peripheral blood monocytes by LPS, and increases the expression of IL-10. Endocrine Soc. 86th Annual Meeting. New Orleans, LA. (Abstract No. P3-524) page 594.
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- 41) Barrett, B.L.* , K.M. Ajuwon*, J.L. Kuske*, and M.E. Spurlock*. 2004. Leptin enhances insulin-stimulated lipogenesis, but does not alter ADP-ribosylation or abundance of the inhibitory G-protein in pig adipocytes. Endocrine Soc. 86th Annual Meeting. New Orleans, LA. (Abstract No. P2-42) pp. 316-317.
- 42) Ajuwon, K.M.* and M.E. Spurlock*. 2004. Adiponectin attenuates the induction of IL-6 by lipopolysaccharide in primary pig adipocytes, and induces the ixpression of PPAR γ 2. Endocrine Soc. 86th Annual Meeting. New Orleans, LA. (Abstract No. P1-64) pp. 169-170.
- 43) Dyson, M.C.* , M. Wulster-Radcliffe*, M. Spurlock*, M. Alloosh, E.A. Mokolke, and M. Sturek*. 2004. Association of interleukin-6 with glucose intolerance and insulin resistance in a swine model of the metabolic syndrome. Endocrine Soc. 86th Annual Meeting. New Orleans, LA. (Abstract No. P2-38) page 316.

d. Papers published in conference proceedings:

- 1) Spurlock, M.E.*, and J.E. Savage*. 1993. Determination of true metabolizable energy content of bobwhite foods. Pages 109-114 in K.E. Church and T.V. Daily eds. Quail III: national quail symposium. Kansas Wildl. And Parks, Pratt.
- 2) Spurlock, M.E.*, M.A. McComb*, and M.A. Roos*. 1996. Effect of weaning and weaning diet on circulating acute phase proteins and intestinal stress protein expression in the pig. Proc. of the 14th Int. Pig Vet. Soc. Cong. Bologna, Italy. pp. 731-737.
- 3) Spurlock, M.E.*, G.R. Frank, S.G. Cornelius, R.P. Chapple, and G.M. Willis. 1996. Impact of environmental factors and disease on animal performance. Proc. of the Minn. Nutr. Conf., Minneapolis. pp. 211-226.
- 4) Spurlock, M.E.*, Weber, T.E.*, and Ajuwon, K.M.*. 2003. New Discoveries in adipocyte and muscle biology: Implications for endocrine and immune regulation of growth in pigs. Proc. Australian Pig Vet. Soc., Cairns, AU. pp. 51-57.
- 5) Spurlock, M.E.*, Jacobi, S.K.*, and Ajuwon, K.M.*. 2004. The role of the adipocyte in energy regulation. Proc. Midwest Swine Nutr. Conf. pp. 51-57.

e. Invited Research Presentations:

I. State

- 1) Leptin: The long and short of a complex story. 2000. Purdue Univ. Interdepartmental Nutrition Cluster. Graduate student recruitment program, keynote address, West Lafayette, IN.
- 2) Leptin, adiponectin, and Toll-like receptors link the adipocyte to the immune response. 2001. Dept. of Vet. Pathobiol., Purdue Univ., West Lafayette, IN.
- 3) Cytokines and Toll-like receptors: The integration of growth and immunology. 2003. Dept. of Basic Medical Sciences, School of Vet. Med. (Dept. seminar), West Lafayette, IN.
- 4) Is there an immunological role for the adipocyte and adiponectin? 2003. Div. of Endocrinol. and Metab. Indiana Univ. School of Med., Indianapolis, IN.
- 5) Adipocytes, inflammation and the metabolic syndrome: Is there a link? 2004. Comparative Medicine Retreat, Purdue Univ., West Lafayette, IN.
- 6) Fat cells, pigs and people: A new look at an old enemy. 2004. School of Agriculture Research Award presentation and seminar, Purdue Univ., West Lafayette, IN.

- 7) The role of the adipocyte in energy regulation. 2004. Midwest Swine Nutr. Conf., Purdue Univ., West Lafayette, IN.

II. National

- 1) Regulation of skeletal muscle and adipose tissue growth and development by cytokines: An overview. 1995. Midwest Meeting, Amer. Soc. of Anim. Sci., Des Moines, IA, Stress and Anim. Growth Symp.
- 2) Leptin: A role in food animal production? 1997. Univ. of Nebraska, Lincoln.
- 3) The cytokinology of leptin. 1997. Amer. Soc. of Anim. Sci. Leptin Symp., Nashville, TN.
- 4) Impact of environmental factors and disease on animal performance. 1997. Minn. Nutr. Conf., Minneapolis, MN.
- 5) The impact of myostatin and selected cytokines on growth. 1998. Amer. Soc. of Anim. Sci. Animal Growth Symp., Denver, CO.
- 6) Leptin and Myostatin: The biology and possible food animal applications. 1998. Univ. of Georgia, Athens, GA.
- 7) Leptin and Myostatin: Targets for embryological manipulation of growth in avian species. 1998. Embrex, Inc., Raleigh, NC.
- 8) The status of leptin and myostatin in food animal biology. 1999. Pfizer Animal Health, Groton, CN.
- 9) Health challenges in food animals: Mechanisms and possible targets for prevention and alleviation. 1999. Univ. of Arkansas, Fayette.
- 10) The impact of disease challenge on growth potential: Recent findings and implications for selected cytokines. 1999. Amer. Soc. of Anim. Sci. Symp., Indianapolis, IN.
- 11) Leptin, leptin resistance, and fat accretion in the pig: Is there a relationship? 2000. Monsanto Co., St. Louis, MO.
- 12) Emerging perspectives on nutrition, body composition and immune function. 2003. Midwest Meeting, Amer. Soc. of Anim. Sci., Des Moines, IA.
- 13) New discoveries in adipocyte and muscle biology: are they new paradigms for growth and nutrition in pigs? 2003. Prince Agri Products, 23rd Annual Feed Ingredient Conf., Rochester, MN.

- 14) Adipocytes and inflammation: The evidence and the implications. 2004. Interdepartmental Nutrition Cluster Symp., The Ohio State Univ., Columbus, OH.

III. International

- 1) Growth potential and disease: A cellular perspective on why sick pigs perform poorly. 1999. American Association of Swine Practitioners Annual Meeting, St. Louis, MO.
- 2) Pigs, nutrition, and technology: Where are things headed? 2000. National Pork Producers Council. World Pork Exp., Pork Academy, Indianapolis, IN.
- 3) Linking energy balance to immune function through leptin and adiponectin. 2002. British Society of Anim. Sci. Symp., York, England.
- 4) New Discoveries in adipocyte and muscle biology: Implications for endocrine and immune regulation of growth in pigs (Co-Keynote Address with Dr. John Black, CSIRO (retired). 2003. Annual Meeting, Australian Pig Veterinary Society. Cairns, Australia. Similar presentations were made at CSIRO research locations in Perth, Jeelong, Toowomba, and Bendigo, and to the Australian Pork Limited technical staff, Canberra.
- 5) Adiponectin, the AMP-activated protein kinase and inflammation in adipocytes. 2004. National Autonomous University of Mexico (UNAM) and the National Institute of Nutrition and Medical Research, Mexico City, Mexico.

f. Patents:

- 1) Porcine Leptin: Sequence and Applications to Pork Production (joint inventor with Dr. Bidwell); #6,277,592.
- 2) Bovine Leptin: Sequence and Applications to Cattle Industries; #6,297,027.
- 3) Porcine adiponectin (pending; File Date: July 2003): Sequence and applications to obesity and inflammation.

g. Other research publications:

- 1) Leininger, M.T., C.P. Portocarrero, C.A. Bidwell, M.E. Spurlock, J.N. Nielsen, and K.L. Houseknecht. 1998. Effect of immune challenge on different genotypes: How sick do they get? Purdue University, Swine Day Report, pp. 1-8.
- 2) Spurlock, M. E., T.E. Weber, and K. Ajuwon. 2003. New discoveries in adipocyte and muscle biology: are they new paradigms for growth and nutrition in pigs? Proceedings of the 23rd Annual Prince Agri Products Feed Ingredients Conf. pp. 4-11.

2. Evidence of Creative Excellence:

Dr. Spurlock's research program can best be described as growth biology with an emphasis on nutrient intake and partitioning, as well as on the metabolic responses of animals to immunological and stress challenges. Additionally, Dr. Spurlock is an active participant in the Comparative Medicine Program, and is working with Dr. Merv Yoder, Indiana University School of Medicine, to establish a myocyte-adipocyte co-culture system using muscle-derived pluripotent cells. New knowledge in these areas is expected to facilitate the development of nutritional, management, and therapeutic strategies that will enhance the efficiency of animal production, and provide novel means of exploring communication pathways between adipocyte and myofibers that will ultimately benefit human health and animal production. He is also collaborating with Dr. Michael Sturek, Indiana University School of Medicine, to develop the Ossabaw pig as a model for the metabolic syndrome in humans. This pig is genetically predisposed to the metabolic syndrome, and progresses to the insulin-resistant pre-diabetic state when allowed ad libitum feed intake. As an additional component of this work, Dr. Spurlock is working with Drs. Moody and Gerrard to introgress the RN gene from Hampshire pigs onto the Ossabaw genetic background. The RN gene is a mutated AMP-activated kinase, and may enhance the utility of the Ossabaw pig as a model for the metabolic syndrome. The following sections describe successful endeavors and specific accomplishments, some of which occurred during Dr. Spurlock's adjunct faculty appointment.

a. The adipocyte:

Recent discoveries have established a new paradigm for the adipocyte, one in which the biological role of this cell extends far beyond the passive storage of excess energy. The discovery of leptin and its strong linkages to metabolic and immunological pathways have helped redefine the concept of the physiological role of adipocytes. These findings, coupled with the knowledge that adiponectin is only produced by the adipocyte and regulates phagocytosis and cytokine production by the macrophage, have provided a strong impetus to explore the linkages of the adipocyte to energy metabolism and immune function. Furthermore, the recent evidence that the adipocyte expresses the receptors for both gram-negative and gram-positive bacteria, and responds to direct stimulation, *in vitro*, underscores the need for knowledge regarding the immunological function of the adipocyte. Dr. Spurlock's research program addresses this new paradigm with emphasis on leptin and adiponectin. He has obtained evidence that leptin acts *directly* on cultured pig adipocytes to stimulate lipolysis, and is currently dissecting the signaling pathway that underlies this response. His work has also shown for the first time that lipopolysaccharide does indeed

stimulate *NFkB-mediated* gene expression and cytokine production in isolated adipocytes, and that adiponectin is a negative regulator of this response to lipopolysaccharide. Furthermore, his laboratory has identified an important differential response in adipocytes vs. macrophages in that cAMP *attenuates* NFkB signaling in macrophages and *stimulates* it in adipocytes. This finding has broad potential implications for cell-specific means of regulating IκBa, the cytosolic inhibitor of NFkB signaling. These two adipocyte-derived proteins may establish a strong integrated linkage among energy metabolism, adiposity, and immune function, and are particularly important to meat animal industries and human health because of the emphasis on reducing adiposity over the past two decades.

b. Leptin:

In collaboration with Dr. Bidwell, Dr. Spurlock's laboratory has focused on the metabolic regulation of leptin expression at the mRNA and protein levels, and the potential role of leptin during the inflammatory response. His group has provided much of the data available to date for the pig, and also developed the research tools required to study the role of leptin in cattle. This research provided some of the first indications of a regulatory relationship between growth hormone, IGF-1, and leptin expression, and also provided considerable evidence that nutritional state, independent of changes in fat mass, is a major determinant of leptin expression. This work has been expanded recently to show that exogenous leptin regulates IGF-1 expression in the liver and serum, independently of feed intake. Recent data from Dr. Spurlock's laboratory indicates that leptin also down regulates PPARγ1 and γ2 in adipose tissue, but has no effect on PPARα in adipose tissue, skeletal muscle, or liver. This points to a marked species difference in pigs (and likely humans) vs. rodent models. The regulation of PPARγ is perhaps casually related to a dedifferentiation event and suppression of lipogenic pathways. Dr. Spurlock has also identified important differences between leptin regulation in rodent models and pigs. Whereas leptin expression is markedly increased in rodents injected with lipopolysaccharide (to simulate bacterial infection), he has shown quite clearly that leptin expression in porcine adipose tissue is not responsive to lipopolysaccharide. This finding raises important questions regarding the potential roles of leptin in the immune response of the pig and the mechanisms by which they are accomplished. Recent evidence from Dr. Spurlock's group has shown for the first time in any species that leptin influences the IgG1:IgG2, and that leptin sustains anti-apoptotic gene expression in activated peripheral blood monocytes, *in vitro*, despite the presence of glucocorticoids. An understanding of the integrated actions of leptin in multiple metabolic states is a critical component of an effective overall strategy to improve growth and efficiency of food producing animals.

c. Adiponectin:

Dr. Spurlock's laboratory has also provided the first data that relate adiponectin production to the innate immune response, *in vivo* and *in vitro*. He is using isolated adipocytes, adipose explants, and 3T3-L1 cells to explore the response of the adipocyte to lipopolysaccharide and specific proinflammatory cytokines. Molecular probes have been constructed to study the expression of the porcine adiponectin gene, and Dr. Spurlock has obtained an antibody to pig adiponectin, and the recombinant protein for *in vitro* use. These research tools are being used to perform the first studies of the regulation of adiponectin

production by leptin, proinflammatory cytokines, and the regulation of adiponectin production in response to lipopolysaccharide, a model of gram-negative bacterial infection.

Recent experiments have resulted in significant findings. First of all, adiponectin blocks the induction of NF κ B signaling in cultured adipocytes stimulated with bacterial endotoxin, but does not influence the response in stimulated myocytes. This points to differential signaling pathways across cell types, and is consistent with the recent discovery of two distinct receptors for adiponectin. Secondly, Dr. Spurlock's laboratory has determined that adiponectin inhibits lipogenesis in cultured pig adipocytes, and has linked this action of adiponectin to the AMP-activated protein kinase. Thus, there are clear immunological and endocrine actions of adiponectin, and these findings support the new paradigm for adipocytes as active immune and endocrine cells. Dr. Spurlock's group has now provided unequivocal evidence that adiponectin induces the expression of interleukin-10 (IL-10) in the macrophage. Because IL-10 is a major anti-inflammatory cytokine, this finding extends the actions of adipocyte-derived adiponectin to include not only suppression of proinflammatory cytokine production, but induction of anti-inflammatory cytokines. Thus, the adiponectin receptor and signaling pathway may provide a means of enhancing circulating IL-10 concentrations, which would be beneficial to animal production and human health.

Finally, Dr. Spurlock's laboratory has recently determined that interferon- γ induces the expression of interleukin-15 in cultured adipocytes, and have now identified key elements of the signaling pathway responsible for this induction. They have now extended this finding to show for the first time in any species that this cytokine acts directly on the adipocyte to regulate lipolysis. This finding is novel, and provides further evidence that the adipocyte regulates nutrient flux because of the well documented regulation of protein accretion in muscle by this cytokine.

d. Growth-stress-cytokine axis:

During periods of immunological challenge and stress, immune and stress modulators orchestrate a reduction in food intake and alterations in peripheral tissue metabolism that diminish growth and repartition nutrients to support higher priority metabolic needs. Reductions in local and systemic concentrations of insulin-like growth factor 1 (IGF-1) may be a critical component of the mechanism by which growth is slowed and nutrients are repartitioned. Dr. Spurlock's laboratory has shown that exogenous leptin reduces IGF-1 expression in the liver of the pig, in parallel with a reduction in serum concentrations. Furthermore, this response is independent of feed intake. These data provide further evidence of a regulatory linkage between leptin and IGF-1. Dr. Spurlock has shown that the proinflammatory cytokines, interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF α), suppress myoblast proliferation and differentiation, and that the effects of these immune modulators are additive. This finding illustrates the potential for disease exposure in the dam to impact the growth potential of her offspring. His group has also provided strong evidence that IGF-1 expression in skeletal muscle is reduced in parallel with serum IGF-1 during infection, independently of feed intake, and despite the administration of exogenous growth hormone. He has extended these findings to also show that serum IGF-1 is reduced in group-housed vs. individually housed pigs, concomitant with a reduction in growth rate and feed intake, and an increase in backfat thickness. These latter findings clearly indicate that energy metabolism and nutrient partitioning are altered by factors as simple as changes in group

size. This interest area has been expanded in Dr. Spurlock's laboratory to encompass the relationships among stress proteins, bacterial endotoxin, and the local production of proinflammatory cytokines in the myocyte as potential causes of the failure of pigs to achieve genetic potential for growth and efficiency in commercial facilities. A recent collaborative arrangement with United Feeds has targeted specific omega-3 fatty acids as regulators of cytokine signaling and growth in both adipocytes and myofibers. Because of access to particular fatty acids through this research relationship, Dr. Spurlock is in a unique position to extend this area of his research and to develop specific nutritional strategies for modulating immune function and growth.

3. Graduate Research Involvement:

a. Completed students:

- 1) Kolapo Ajuwon, Regulation of growth and metabolic markers in peripheral tissues of the pig by exogenous leptin, M.S. (Co-advisor with O. Adeola), 2001. (Publication #31, 32, page 6; Abstract #35, page 10).
- 2) Mahogany Wade, MS. (non-thesis), 2001.
- 3) Thomas Weber, The effects of exogenous leptin on immunological variables of growing pigs and leptin effects upon cultured monocytes and T-cells, Ph.D., 2003. (Publication #35, page 6; Abstract #37, page 10).

b. Current students:

- 1) Kolapo Ajuwon, Ph.D. (candidate) Immunological roles of the adipocyte and adipocytokines. (Publication #34, 38, page 6; Abstract #41, 42, page 10).
- 2) Sheila Jacobi, Ph.D. (candidate) Endocrine activity of the porcine adipocyte. (Publication #37, page 6).
- 3) Sun Hye Shin, Ph.D. (co-advised with Dr. Gerrard). Intercellular communication between adipocytes and myocytes.

c. **Advisory committee member:**

Student	Degree	Major Professor	Dept.	Date of Degree
Chavis, Christy	M.S.	D. Hancock	ANSC	1994
McComb, Molly	M.S.	A.L. Grant	ANSC	1997
Leininger, Michael	M.S.	K. Houseknecht	ANSC	1999
Weber, Thomas	M.S.	A.P. Schinckel	ANSC	2000
McKee, Carrie	M.S.	S. Eicher	ANSC	2001
Velez, Juan Carlos	M.S.	S. Donkin	ANSC	2002
Norberg, Sarah	M.S.	M.A. Latour	ANSC	2002
Durkin, Rachel	M.S.	R. Krisher	ANSC	2003
Crowder, Stacie	M.S.	S.S. Donkin	ANSC	2003
Trapp, Scott	M.S.	B. Richert	ANSC	2003
Herrick, Jason	Ph.D.	R. Krisher	ANSC	2004
Johnson, Trina	M.S.	S. Eicher	ANSC	2004
Williams, Liz	M.S.	Donkin	ANSC	2004
Zhu, Harry	M.S.	D. Moody	ANSC	2004
Dilger, Anna	M.S.	D.E. Gerrard	ANSC	2004
Selig, Kristin	M.S.	A. Bhunia and J. Patterson	ANSC	2004
Arseneau, Jeff	Ph.D.	R.P. Lemenager	ANSC	Current
Follas, Dan	Ph.D.	J. A. Christian	VPB	Current
Hazleton, Sarah	Ph.D.	S. S. Donkin	ANSC	Current
Schreiweiss, Melissa	Ph.D.	P.Y. Hester	ANSC	Current
Cho, Kae Won	Ph.D.	Y.-C. Kim	F&N	Current
Karcher, Darrin	Ph.D.	T. Applegate	ANSC	Current
¹ Camacho, Maria del Carmen	Ph.D.	Rogelio Alonso	Molecular Genetics	Current

¹Co-advised with Dr. Rogelio Alonso, Department of Molecular Genetics, National Autonomous University, Mexico City, Mexico

4. Postdoctoral Research Associates:

- 1) Shaoquan Ji. 1996-1999. Dr. Ji worked under Dr. Spurlock's direction at Purina Mills, Inc., and is currently a staff scientist at Linco Research, Inc., St. Louis, MO. (Publication #12-14, 17-18, 20, 26, pages 4-5; Abstract #9, 11, 13, 17, 18, 20-22, 30, pages 7-9).
- 2) Priya Raman. 2000-2002. Dr. Raman had responsibility for a collaborative project with Dr. Donkin that was designed to identify specific pathways in the hepatocyte by which leptin influences energy substrate utilization. She is currently employed at the Indiana University School of Medicine, Diabetes Center, as a Sr. Research Investigator. (Publication #33, page 6; Abstract #34, page 10).
- 3) Meghan Wulster-Radcliffe. 2003-2004. Dr. Wulster-Radcliffe currently has had responsibility for the macrophage-adiponectin objective in the grant funded by the Biotechnology Research & Development Corp., but is now employed by Eli Lilly & Co. (Publication #36, b1, page 6; Abstract #39, 40, 43, page 10).

- 4) Nick Gabler. 2004-Present. Dr. Gabler has major responsibility for a joint project with United Feeds, Inc. that involves the regulation of immune response pathways by fatty acids.

5. GRANT ACTIVITIES (Research grants and awards received)

5.1. Current Grants

1. Agency/Title of Grant: Showalter Foundation. A porcine model of the metabolic syndrome.
 2. Duration of Funding: 7/1/04 to 6/30/05
 3. Total amount of award: \$75,000
 4. Your role: PI (Co-PI's Drs. Moody and HogenEsch)
 5. If Co-PI, for how much of the total funding are you directly responsible: \$60,000
-

1. Agency/Title of Grant: United States Department of Agriculture – National Research Initiative. Regulation of lipogenesis and lipolysis in porcine adipocytes by leptin.
 2. Duration of Funding: 9/15/01 to 9/30/04
 3. Total amount of award: \$175,000
 4. Your role: PI
 5. If Co-PI, for how much of the total funding are you directly responsible: _____
-

1. Agency/Title of Grant: United States Department of Agriculture – National Research Initiative. Regulation of lipogenesis and fatty acid oxidation in the pig by adiponectin.
 2. Duration of Funding: 10/1/03 to 9/30/06
 3. Total amount of award: \$240,000
 4. Your role: PI
 5. If Co-PI, for how much of the total funding are you directly responsible: _____
-

5.2. Pending Grants

- | | | |
|----|---|--|
| 1. | Agency/Title of Grant: | United State Department of Agriculture – National Research Initiative. Regulation of cytokine production and inflammation in porcine adipocytes by adiponectin and the ppar transcription factors. |
| 2. | Duration of Funding: | 1/1/05 to 12/31/07 |
| 3. | Total amount of award: | \$260,251 |
| 4. | Your role: | PI |
| 5. | If Co-PI, for how much of the total funding are you directly responsible: | |

- | | | |
|----|---|---|
| 1. | Agency/Title of Grant: | National Institutes of Health. Characterizing a new pig model of the metabolic syndrome. |
| 2. | Duration of Funding: | 1/1/05 to 12/31/09 |
| 3. | Total amount of award: | \$1,879,199 |
| 4. | Your role: | PI, Co-PIs are Drs. Moody and Gerrard, Animal Sciences, Mittal, Veterinary Pathobiology and Sturek, IU School of Medicine |
| 5. | If Co-PI, for how much of the total funding are you directly responsible: | \$964,700 |

- | | | |
|----|---|---|
| 1. | Agency/Title of Grant: | American Diabetes Association. Obesity-related changes in inflammatory status and adiponectin receptors in adipocytes and macrophages, and potential links to the metabolic syndrome. |
| 2. | Duration of Funding: | 1/1/05 to 12/31/07 |
| 3. | Total amount of award: | \$300,000 |
| 4. | Your role: | PI |
| 5. | If Co-PI, for how much of the total funding are you directly responsible: | |

5.3. Past Grants

1. Agency/Title of Grant: Monsanto-Dekalb, St. Louis, MO. Potential biological and molecular indicators of growth and body composition in two commercial sire lines.
 2. Duration of Funding: 4/1/00 to 6/30/02
 3. Total amount of award: \$174,300
 4. Your role: PI
 5. If Co-PI, for how much of the total funding are you directly responsible:
-

1. Agency/Title of Grant: Monsanto-Dekalb, St. Louis, MO. Growth and efficiency of two commercial sire lines.
 2. Duration of Funding: 5/15/00 to 11/30/00
 3. Total amount of award: \$32,303
 4. Your role: Co-PI with Drs. Richert and Schinckel
 5. If Co-PI, for how much of the total funding are you directly responsible: \$6,000
-

1. Agency/Title of Grant: Purdue Agricultural Research Programs Assistantship. Metabolic and immunological responses to bacterial infection: a comparison of genotypes.
 2. Duration of Funding: 1/1/00 to 12/31/02
 3. Total amount of award: \$30,000
 4. Your role: PI
 5. If Co-PI, for how much of the total funding are you directly responsible:
-

1. Agency/Title of Grant: Fats and Protein Research Foundation, Inc. Evaluation of the effects of dietary fat, ractopamine and conjugated linoleic acid (cla) on growth performance and pork quality in genetically learn gilts.
 2. Duration of Funding: 9/1/00 to 6/30/02
 3. Total amount of award: \$39,332
 4. Your role: Co-PI with Drs. Schinckel, Richert, Forrest and Dr. John Eggert, Continental Grain, Inc.
 5. If Co-PI, for how much of the total funding are you directly responsible: \$1,100
-

1. Agency/Title of Grant: Purdue Research Foundation Graduate Fellowship. The importance of adiponectin and leptin to the innate immune response in the pig.
 2. Duration of Funding: 1/1/02 to 12/31/03
 3. Total amount of award: \$25,292
 4. Your role: PI
 5. If Co-PI, for how much of the total funding are you directly responsible:
-

1. Agency/Title of Grant: Showalter Foundation. Functional genomics for animal research.
 2. Duration of Funding: 7/1/01 to 6/30/02
 3. Total amount of award: \$83,000
 4. Your role: Co-PI with Drs. Bidwell and Moody
 5. If Co-PI, for how much of the total funding are you directly responsible: \$21,000
-

1. Agency/Title of Grant: Biotechnology Research and Development Corporation.
Adiponectin: a novel regulator of immune function and
energy metabolism.
2. Duration of Funding: 1/1/02 to 4/30/03
3. Total amount of award: \$59,451
4. Your role: PI
5. If Co-PI, for how much of the total funding are you directly responsible: _____

1. Agency/Title of Grant: Biotechnology Research and Development Corporation.
Regulation of inflammation by a novel adipocyte protein.
2. Duration of Funding: 1/1/02 to 12/31/03
3. Total amount of award: \$269,000
4. Your role: PI
5. If Co-PI, for how much of the total funding are you directly responsible: _____

Summary of Funding

Year	Total Amount of Grant	Amount of Grant to Dr. Spurlock	Gift in Kind	Total Amount to Dr. Spurlock & Purdue University
2000	\$275,935	\$194,500	\$30,000	\$224,500
2001	\$258,000	\$196,000	\$0	\$196,000
2002	\$353,743	\$84,743	\$5,750	\$90,493
2003	\$240,000	\$240,000	\$8,800	\$248,800
2004	\$75,000	\$60,000	\$4,300	\$64,300
Total	\$1,202,678	\$775,243	\$48,850	\$824,093

b. Gifts:

- 1) Pig Improvement Co., \$30,000. 2000.
- 2) Nestle-Purina, \$18,850. 2002, 2003, 2004.
- 4) United Feeds, Inc. \$45,000. 2004.

6. Agriculture Research Programs (ARP) Project:

ARP Project 51066, Endocrine actions of adipocytes in the control of energy balance and immune function.

7. Evidence of Interdisciplinary Activity:

- a. Collaborative effort with Dr. Chris Bidwell to produce leptin research reagents to support metabolic and regulatory studies. Current work includes the development of cell populations for SAGE analysis. Four manuscripts (#11, 13, 14, 25, pages 4-5).
- b. Collaborative effort with Dr. Shawn Donkin to identify the signaling pathways by which leptin controls energy metabolism in isolated pig hepatocytes. One manuscript (# 33, page 6).
- c. Collaborative effort with Dr. Rebecca Krisher to explore the role of leptin and the leptin receptor in oocyte maturation and embryonic development, pre-implantation.
- d. Collaboration with Dr. Diane Moody to test the efficacy of human microarrays for pig tissues. Models include *in vivo* and *in vitro* exposure of adipocytes to leptin and lipopolysaccharide. Two Showalter grants funded, NIH and 21st Century grants pending.
- e. Collaboration with Dr. Karen Houseknecht to investigate the relationship among dietary fatty acid profile, leptin, and peroxisome proliferator activated receptors. Five manuscripts (#19, 22, 23, 25, 28, page 5) and five abstracts (#25, 28, 29, 31, 32, page 9).
- f. Collaborative studies with Dr. Alan Grant in the regulation of IGF-1 during immune challenge and stress, and in response to leptin. One manuscript (#14, page 4) and three abstracts (#6, 14, 15, pages 7-8).
- g. Collaboration with Drs. Dave Gerrard and Scott Mills to establish *beta*-adrenoceptor density in porcine oxidative and glycolytic muscle, and the influence of ractopamine on these receptors. Also collaborated with Dr. Gerrard to show that myostatin mRNA was localized to the myofiber and secretory epithelial cell in mammary gland. One manuscript (#17, page 4) and one abstract (#6, page 6); NIH grant pending.
- h. Collaboration with Dr. Scott Mills pertaining to the regulation of lipogenic genes by dietary fat and growth hormone, and regulation of lipolysis by β -adrenoceptor agonists. One manuscript (#29, page 5).

- i. Collaboration with Dr. Rex Gaskins, University of Illinois. Past projects have been designed to help understand the immunological role of the small intestine in the weanling pig. One manuscript (#21, page 5).
- j. Collaboration with Dr. Bob Matteri, USDA-ARS, to study the regulation of the leptin receptor in porcine adipose tissue and skeletal muscle. Dr. Matteri has accepted a new position within the USDA-ARS, and Dr. Spurlock has assumed full responsibility for this project.
- k. Collaborative project with Dr. Jess Miner, University of Nebraska, to identify the regulatory roles of adipisin, with an emphasis on the adipocyte. Two manuscripts (#8, 27, pages 4 & 5).
- l. Collaboration with Dr. John Christian, Purdue University School of Veterinary Medicine, to establish procedures for deriving functional macrophages from peripheral blood monocytes. One manuscript (#36, page 6) and one abstract (#39, page 10).
- m. Collaboration with Dr. Merv Yoder, Indiana University School of Medicine, to establish a co-culture system for adipocytes and myofibers using a pluripotent cell of muscle origin.
- n. Collaboration with Dr. Suresh Mittal, Purdue University School of Veterinary Medicine, to use adenovirus technology to study adiponectin in pigs (USDA-NRI funded, 2003; NIH grant pending).
- o. Collaboration with Dr. Rogelio Alonso, Dept. of Molecular Genetics, National Autonomous University of Mexico (UNAM), Mexico City, Mexico. This project entails a physiologic and genetic comparison of the Mexican Hairless Pig and the Cuino as potential models of obesity and Type II diabetes. Dr. Spurlock has been admitted to the graduate faculty at UNAM, and he and Dr. Alonso are co-advising a Ph.D. student on this project.
- p. Collaboration with Dr. Anne Reifel-Miller, Eli Lilly & Co. Dr. Reifel-Miller has provided PPAR ligands and adiponectin receptor adenoviral constructs, and will be providing assays for a pending Diabetes grant.
- q. Collaboration with Dr. Mike Sturek, Indiana University School of Medicine, to develop the Ossabaw pig as a model for the metabolic syndrome. 21st Century and NIH grants pending.

8. Other Evidence of Recognition:

a. Peer Review Contributions to Scientific Journals:

- 1) Member of editorial board, *Journal of Animal Science-Nonruminant Nutrition* (1998-2001).
- 2) Member of editorial board, *Domestic Animal Endocrinology* (2000-2003).
- 3) School of Agriculture Nominee, David and Lucille Packard Research Fellowship Competition (2003).

- 4) Invited contributor, *Nutrition Reviews International*.
- 5) Member of editorial board, *Journal of Animal Science*-Growth and Development (2004-2007).
- 6) *Ad hoc* reviewer for the following journals:
 - a) *American Journal of Physiology*
 - b) *Journal of Interferon & Cytokine Research*
 - c) *Hepatology*
 - d) *Physiology & Behavior*
 - e) *Nutritional Biochemistry*
 - f) *Journal of Nutrition*
 - g) *Journal of Endocrinology*

b. Grant Review Activity:

Dr. Spurlock serves as an ad hoc reviewer for the United States Department of Agriculture National Research Initiative Competitive Grants Program. Since 1996, he has reviewed an average of five proposals per year for the Growth and Development, Genetic Mechanisms and Animal Health and Well-Being divisions. Dr. Spurlock also serves as an ad hoc reviewer for the National Institutes of Health, Adipocyte Biology and NIDDK sections, and for the Veterans Administration, Gerontology section.

c. Contributions to American Society of Animal Science:

- 1) Midwest section, member of program committees
Nonruminant Nutrition (1996-1998).
Growth and Development (1999-2001; Chair, 2001).
- 2) Midwest section meetings, scientific session chair in 1996, 1997, 1998 and 1999.
- 3) National ASAS meetings, scientific session chair in 1996, 1998, and 1999.
- 4) National ASAS Triennial Growth Symposium Committee (2003-2004).

d. Advisory Board Memberships:

- 1) Monsanto-DeKalb; appointed to team focused on improving the efficiency of feed utilization in commercial genetic lines (2000-2003).
- 2) Pig Improvement Company (PIC); appointed to research advisory board charged with designing the research strategy to increase the realization of genetic potential in PIC genetic lines (1999-2003).
- 3) AusGene; member of an academic consulting team led by Dr. Terry Stewart to address issues related to the optimal selection of genetically superior animals (2000 to present).

- 4) Appointed *ad hoc* member, Research Advisory Board, School of Veterinary Medicine, Purdue University (2002-2005).

e. Faculty Mentoring:

- 1) Dr. Spurlock is currently acting as a mentor in support of a K01 grant for Dr. Ignacio Camarillo, Dept. of Biology, Purdue University. This is an NIH grant that requires joint laboratory activities between Drs. Spurlock and Camarillo, and development of an integrated project. This grant targets the relationship between adipocytes and mammary epithelial cells in mammary tumorigenesis.

B. EXCELLENCE IN TEACHING

Even though Dr. Spurlock does not have a primary teaching appointment, he taught ANSC 324 (Animal Nutrition), a very important course in the curriculum, for two years. This is one of three courses that will fulfill the advanced nutrition requirement for Animal Sciences majors and typically has an enrollment of 85-100 students. Dr. Spurlock's approach to this course, and to teaching in general, was to present the facts, develop the concepts, and then guide the students through the practical applications of the knowledge. The ANSC 324 course emphasizes the application of nutrition principles to the formulation and preparation of diets that adequately meet the nutrient needs of animals. Considerable effort is devoted to helping students grasp the fundamentals of computerized diet formulation, using software that is common to the commercial feed and production industries. Laboratory assignments and homework problems are used to cultivate nutritional expertise. The students are challenged to apply their knowledge to carry out a research project in which they work individually and in teams to formulate and mix diets to accomplish an assigned nutrition objective. The students feed and care for their chickens for a period of two weeks. Data are collected, analyzed statistically, and reported in journal article (Poultry Science Journal or Journal of Nutrition) format.

Dr. Spurlock also introduced two new focal points for the course. First, two laboratory periods are now devoted to the practice of feed microscopy. Students are taught to recognize specific feed ingredients via their inherent microscopic characteristics, and are instructed as to the potential uses and limitations of feed microscopy from a production and regulatory perspective. Secondly, one week (lecture and laboratory) is now devoted to companion animal nutrition. Dr. Spurlock collaborated with research and technical service scientists from the Ralston Purina Company to conduct the sessions that identified unique nutrient needs of companion animals, in addition to reading and understanding pet food labels. Also, concepts of prophylactic and therapeutic nutrition were introduced in conjunction with the potential for specific nutrients and feed ingredients to impact physiological processes.

Dr. Spurlock has also developed an adipocyte biology section for the new course, FN/ANSC 595. This module has been taught for two years, and has led Dr. Spurlock to develop and teach a new graduate level adipocyte biology course (ANSC 595R) that covers basic aspects of energy metabolism in adipocytes, but focuses largely on the newly discovered role of the adipocyte as an endocrine and immune cell. This course was initiated in the Fall semester, 2003. Special emphasis is placed on biochemical pathways relating to energy utilization and regulation of immune function in

light of the hormones, growth factors, and cytokines produced by the adipocyte, and the tissues bearing receptors to these regulatory factors. The initial evaluation of this course by graduate students was excellent. Dr. Spurlock will teach this course for the second time in the Fall of 2004.

1. Courses taught:

Course	Semester, year	Credits	Number of Students
ANSC 324	Spring, 2001, 2002	3	87
ANSC 595R	Fall, 2003	2	12

2. Student evaluations:

Course	Year	No. of Responses	University Core ^a		Departmental Core ^b					
			1	2	1	2	3	4	5	6
ANSC 324	2001	74	4.2	4.7	4.7	4.7	4.4	4.2	4.1	4.3
ANSC 324	2002	73	4.0	4.6	4.3	4.6	3.9	4.0	4.1	4.2
ANSC 595R	2003		4.7	4.9	4.9	5.0	4.6	4.9	5.0	4.8

^aUniversity Core Questions (strongly agree=5):

Overall, I would rate this course as Excellent-Good-Poor-Very Poor

Overall, I would rate this instructor as Excellent-Good-Poor-Very Poor

^bDepartmental Core

1. My instructor seems well prepared for class.
2. Students are encouraged to see the instructor if they are having difficulty.
3. My instructor gives exams, which accurately reflect the course material.
4. The climate of this class is conducive to learning.
5. This course effectively challenges me to think.
6. This course builds understanding of concepts and principles.

C. EXCELLENCE IN EXTENSION AND SERVICE

Dr. Spurlock does not have a formal extension appointment, but participates in activities as requested. He was a speaker at Purdue's 80th annual Swine Day (2000) and has participated on several committees.

1) Service on School Committees:

- a. Roadmapping and Strategic Planning Committee, 2002
- b. Technology transfer subcommittee, 2001
- c. Relationship development, Purdue and Dow AgroSciences, 2003 to present
- d. Agriculture Life Sciences Summit, 2004
- e. Dean of Agriculture Search Committee, 2004

2) Service on Departmental Committees:

- a. Meats Laboratory and Facilities Committee, 1999-2002
- b. Renovation of Lilly Animal Facility Committee, 2000
- c. Louja Graduate Student Travel Award Committee, 2000
- d. Swine Programs Committee, 2000
- e. Swine Day Planning Committee, 2000
- f. Chair, Growth and Development Committee for Cooperative State Research, Education and Extension Service (CSREES) Review, 2001
- g. Chair, Nutrient Utilization Search Committee, 2001
- h. Department Head Search Committee, 2001
- i. Graduate Committee, 2001-2003
- j. Interdepartmental Nutrition Programs Admissions Committee, 2001-2003
- k. Advisor, Block & Bridle Club, 2001-2003
- l. Chair, Research Strategic Planning Team, 2002-2003
- m. Chair, Departmental Seminar Committee, 2002

3) Professional Improvement Activities:

- a. International conference and workshop on proteases, Tokyo, Japan, 1994
- b. Growth biology techniques workshops, Minneapolis, MN, 1994
- c. University of Florida-USDA grantmanship workshop, Orlando, FL, 1999
- d. "Writing a Successful NIH or USDA Grant" workshop, Purdue University, West Lafayette, IN, 2000
- e. ESCOP/ACOP Leadership Program (Class 13, Phase), West Lafayette, IN; Indianapolis, IN; Washington, DC. 2003-2004

SUMMARY OF SUPPORTING STRENGTHS

Page(s)

I. Evidence of Excellence in Research	
A. Developed a focused research program to uncover strategies to improve the efficiency of meat animal production.	2, 14-17
B. Recipient of the School of Agriculture Research Award.	2
B. Authored or co-authored 41 manuscripts in 13 different peer-reviewed journals, 5 manuscripts in conference proceedings and 43 published abstracts.	3-11
C. Awarded two patents, with a third pending.	13
D. Obtained \$1,202,678 (directly responsible for \$824,000) in research support.	19-23
E. Currently has three Ph.D. students and has guided three post-doctoral scientists.	17-18
F. Collaborates effectively with several other scientists.	24-25
II. Evidence of Excellence in Teaching	
A. Received excellent student evaluations in Animal Nutrition (ANSC 324) and Adipocyte Biology (ANSC 595R).	28
B. Participates in an adipocyte module for course for Animal Sciences and Foods & Nutrition graduate students, and has developed a graduate level adipocyte biology course.	27
III. Evidence of National and International Recognition	
A. Twenty-eight invited research talks in domestic and international forums.	11-13
B. Member of editorial boards of two prestigious journals concurrently.	25
C. Serves as ad-hoc reviewer for seven additional scientific journals.	26
D. Serves as an ad-hoc reviewer for three divisions of the USDA-NRICGP, two sections of the NIH, and one section of the Veterans Administration.	26
E. Is (or has been) a member of four advisory boards.	26-27
F. Invited to graduate faculty at UNAM; invited collaboration on government research project to compare the metabolic and genetic aspects of two potential pig models of obesity and diabetes.	25

SUPPORTING LETTERS – DR. MICHAEL E. SPURLOCK

- **Dr. Cliff Baile** holds the titles of Distinguished Professor of Animal Sciences and Foods and Nutrition and Eminent Scholar in Agricultural Biotechnology at the University of Georgia.

Dr. Baile is acquainted with Dr. Spurlock's research through professional and collaborative interactions while Dr. Spurlock was at Purina Mills, Inc., and at Purdue University

- **Dr. Rodney Johnson** is currently Professor, Integrative Biology, Department of Animal Sciences, University of Illinois.

Dr. Johnson is a world renowned expert in the relationship between growth and the immune system. His laboratory has done pioneering work to define the regulatory linkages between disease and the molecular regulation of cytokine production within the central nervous system. Dr. Johnson and Dr. Spurlock have been joint speakers at multiple conferences over the past 10 years.

- **Dr. Jack Odle** is currently Professor of Nutrition, Department of Animal Sciences, North Carolina State University.

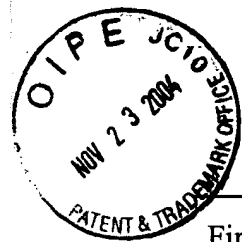
Dr. Odle's program is focused in the area of neonatal nutrition and metabolic regulation. Emphasis is on developmental aspects of lipid digestion, absorption and metabolism at the molecular, cellular and whole-animal level. Dr. Odle is an extremely well-published scientist, and has served as the associate editor for the Journal of Nutrition.

- **Dr. Karen Houseknecht** is currently a Sr. Research Leader at Pfizer's Central Research Division in Groton, Connecticut.

She has responsibility for the group that leads the company's research efforts in the phenotypes common to the metabolic syndrome. She has collaborated extensively with Dr. Spurlock and her expertise in metabolic diseases is widely recognized.

- **Dr. Harm HogenEsch** is currently Professor and Head, Department of Veterinary Pathobiology, School of Veterinary Medicine, Purdue University, and is also the director of the Center of Excellence in Comparative Medicine at Purdue University.

Dr. HogenEsch has served on doctoral student committees with Dr. Spurlock, and has had numerous interactions via the comparative medicine program.



First Named Inventor : Michael E. Spurlock	
Appln. No. : 09/932,888	
Filed : August 20, 2001	Group Art Unit: 1647
Title : Porcine Leptin Protein, Antisense and Antibody	Examiner: C. J. Saoud
Docket No. : LL31.12-0016	

EXHIBIT B

of

DECLARATION

submitted under 37 C.F.R. 1.132

Rawl, J. David, Biochemistry, Pages 993-994
Carolina Biological Supply Company, 1989

Biochemistry

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clone from another type of organism, such as wheat. In this latter instance, the DNA sequences need not be identical but should be about 60 to 70% homologous since the conditions under which nucleic acid hybridization occurs can be manipulated to detect either partially homologous sequences or a perfectly matched duplex. For highly conserved genes, such as those for histones, the conserved sequences allow hybridization among even distantly related species.

Another hybridization technique, called *chromosome walking*, also called *overlap hybridization*, is a technique for isolating overlapping recombinants in order to "walk" from one position, usually a previously cloned and analyzed gene, to another position nearby on the chromosome. This technique, as outlined graphically in Figure 30-11, begins by isolating a terminal fragment from the starting recombinant. This terminal fragment is labelled and used as a hybridization probe for a genomic library, where it will identify all recombinants that contain it. These recombinants are then mapped with restriction endonucleases to identify those that overlap with the probe and extend into the region adjacent to the starting recombinant. The terminal fragment of this newly isolated recombinant is then used as the probe for the second "step" in the walk. This process is continued until the walk reaches the desired destination.

The nature of the destination site determines how arrival at it is identified. For example, in yeast, chromosome walking was used to move from the *LEU2* gene to a closely linked site on chromosome 3, the *centromere*. Since the centromere maintains proper segregation of chromosomes during mitosis, its presence on a recombinant was tested functionally by its ability to maintain proper segregation of plasmids in yeast cells during mitosis. In *Drosophila*, chromosome walking is routinely used to isolate genes whose function is unknown but whose physical position on the chromosome is known through detailed genetic studies. In these cases, the assay for successful completion of the walk is hybridization to the proper chromosomal band on a polytene chromosome isolated from salivary glands (Chapter 24). *Drosophila* geneticists have also used chromosome walking to isolate a number of very large (50 kbp and larger) genes. These genes are often interrupted by such large introns that screening a genomic library with a cDNA probe can identify two or more recombinants, each of which contains complementary sequences but neither of which is contiguous on the chromosome and neither of which includes the entire gene. Chromosome walking allows these noncontiguous recombinants to be linked, thereby establishing the complete structure of the gene.

Box 30-1 Nucleic Acid Hybridization

The specificity with which DNA and RNA molecules form stable duplex structures leads to the powerful application of this property in the technique known as *nucleic acid hybridization*. Hybridization means base pairing, and the technique is used to identify and determine the location of specific nucleic acid sequences within a larger sequence, such as a genome, recombinant DNA molecule, or mixture of RNA molecules. The stability of the duplex is directly related to the complementarity of the two nucleic acid strands. Physical conditions under which hybridization is carried out can be manipulated (referred to as altering the stringency) in order to use one nucleic acid strand as a probe for its complementary partner. Under the most stringent conditions, which include high temperature and low salt concentration, a probe sequence will hybridize only to its perfect complement. As the stringency is lowered, mismatches between the strands can be tolerated without destabilizing the

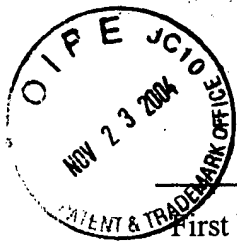
duplex. Hybridization under lower stringency is thus useful for detecting desired sequences that are partially similar but not identical, such as might be encountered when working with probe DNA and target DNA from different species. If the stringency is too low, nucleic acids will hybridize over several very short complementary regions, and specificity will be lost. A number of related techniques have been developed to apply the property of nucleic acid hybridization to screening for and isolation of specific sequences. Most of these techniques operate with a replica of the DNA of interest immobilized on a solid support, such as a nylon or nitrocellulose membrane. One of the first such methods, developed in the mid 1970's, became known colloquially as *Southern blotting*, named after its developer, Ed Southern.

In the Southern blotting procedure (Figure 1), DNA is digested with one or more restriction endonucleases and the resulting fragments separated by electrophoresis through an agarose gel. The double-stranded DNA fragments are visualized by staining, denatured in situ by soaking the gel in sodium hydroxide, and transferred to the membrane by capillary transfer in a high-concentration salt solution. These conditions allow the DNA to be retained on the filter at the point of contact between gel and filter, thus creating a replica of the gel. The DNA is then covalently bound to the filter using heat or ultraviolet light. Hybridization of probe nucleic acid (radioactively labelled DNA or RNA can be used) to the denatured DNA on the filter is carried out under the desired stringency, and after washing the excess unbound probe from the filter, the position at which specific binding occurred can be detected by autoradiography of the filter. The hybridization pattern can then be compared directly to the region of the original gel (one or a few bands) that contains the DNA sequences of interest. Southern blotting is such a powerful technique because it extends the information gained by making a restriction map of a particular piece of cloned DNA, for example, and identifies the region of that DNA that contains the sequence of interest. The Southern blotting procedure also enables nucleic acid probes to be used as diagnostic tools for many genetic disorders by probing genomic DNA from affected individuals and family members.

Following Southern's procedure, related techniques for probing DNA in situ from recombinant *E. coli* colonies (developed by M. Grunstein and D. S. Hogness) and bacteriophage plaques (developed by W. D. Benton and R. W. Davis) have been devised. In addition, J. C. Alwine, D. J. Kemp, and G. R. Stark have developed a related technique, which differs in that the gel electrophoresis fractionates not DNA but RNA. Because this technique is so similar to Southern blotting, it is known colloquially as Northern blotting. These techniques have exploited the powerful property of nucleic acid hybridization in the development of recombinant DNA technology.

B. Some Nucleic Acid Probes Are Derived from Amino Acid Sequences

Another type of information commonly available is the partial amino acid sequence of peptides generated from the protein of interest. From this information, the genetic code can be used to derive several nucleotide sequences corresponding to all possible mRNA sequences that could encode the peptide. One of these



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Special Report

Fundamentals of quality assessment of molecular amplification methods in clinical diagnostics

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▶ Abstract

The increasing interest in molecular biology diagnostics is a result of the tremendous gain of scientific knowledge in genetics, made possible especially since the introduction of amplification techniques. High expectations have been placed on genetic testing, and the number of laboratories now using the relevant technology is rapidly increasing—resulting in an obvious need for standardization and definition of laboratory organization. This communication is an effort towards that end. We address aspects that should be considered when structuring a new molecular diagnostic laboratory, and we discuss individual preanalytical and analytical procedures, from sampling to evaluation of assay results. In addition, different means of controlling contamination are discussed. Because the methodology is in constant change, no general standards can be defined. Accordingly, this publication is intended to serve as a recommendation for good laboratory practice and internal quality control and as a guide to

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troubleshooting, primarily in amplification techniques.

► Introduction

The following recommendations on quality assessment of molecular biology methods in clinical diagnostics refer to the preanalytical and analytical steps, particularly those of amplification techniques. In particular, the polymerase chain reaction (PCR) and methods based thereon are those primarily used for developing laboratory tests that have potential for future routine applications. We have not attempted to deal with individual applications, which are at present in constant change and evolution and for many of which the clinical significance has yet to be established.

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Because of the special requirements associated with amplification techniques, the aspects of good laboratory practice dealt with in depth are those regarding preanalytical and analytical aspects of nucleic acids amplifications. Many of the recommendations are aimed at avoiding contaminations and facilitating timely recognition of contaminants, should they occur. Although every amplification assay is prone to contamination, the technical effort associated with different types of nucleic analyses varies widely. For example, genotyping patients usually does not require optimization of the amplification conditions for improved detection limits. On the other hand, for detection of minimal residual disease or for virus detection, a very low detection limit is prerequisite. Accordingly, contamination risk may be not a major problem in one application but obviously can be critical in another. Also, if different enzymes have to be used in subsequent steps of a given test, as in reverse transcriptase (RT) PCR, the handling of the additionally required material must be considered a potential contamination hazard.

The potential of contamination is especially serious if, within the scope of diagnosis, the same DNA sequence is amplified repeatedly, or if amplification products are subjected to additional rounds of amplification, as exemplified in so-called seminested or nested PCR procedures. In nested PCR, the specificity of amplification can be enhanced through use of a second set of internal primers. As in "one-step" PCR, an excessive number of amplification cycles will generate nonspecific signals. In addition, nested assays are particularly prone to contamination because the PCR products generated during the first round of amplification are usually pipetted into new reaction tubes before reamplification. Alternatively, execution of nested PCR in a single reaction vessel, where possible, is preferable to the usual nested PCR approach (1)(2). In general, the gain in specificity by reamplification of the products of the first round should be weighed against the increased risk of contamination. At present, the use of nested PCR application cannot be generally recommended for clinical diagnostics but should be performed only by experienced laboratories.

Regardless of the amplification system being considered, the possibility of spreading the amplification products by aerosols into other reaction vessels as a source of contamination always has to be a general concern. Avoidance of contaminations requires careful planning of preanalytical and analytical steps. For the purpose of these fundamental recommendations, we presume that a molecular diagnostic

laboratory using amplification methods will provide a test program with assays that differ in their detection limit requirements, e.g., HLA typing and virus detection in blood specimens. The precautions taken should always reflect the needs of the most critical assay and should aim at the highest quality with respect to the test program. The laboratory staff must be clearly aware of the consequences of inadequate performance and quality control, and appropriate training must be given within the laboratory to ensure the high skills needed for molecular analysis. Once a contamination of reagents or stock solutions has occurred, it is often very difficult and time-consuming to localize and eliminate its source; consequently, all of the reagents may have to be discarded and replaced. Therefore, the aspects of laboratory organization and work flow discussed here are intended to assist the staff in preventing contaminants and to eliminate potential sources thereof. However, because danger of contamination cannot be entirely disregarded, even in the best circumstances, appropriate internal quality control is absolutely essential.

The current recommendations aim to define good laboratory practice rules for molecular biological methods. For the reasons given above, we do not differentiate between the more error-prone methods and those that are less so. We particularly emphasize techniques that utilize PCR, the most widely used method in the emerging field of nucleic acids diagnostics. However, most of the aspects that apply to PCR are also valid for other target amplification techniques.

► 1. Laboratory Organization and Laboratory Equipment

The problems associated with the avoidance of contamination in PCR necessitate a decisive and strictly-adhered-to laboratory organization, including room and space planning. Ideally, a PCR laboratory should be divided into four separate work areas, each having dedicated special equipment for: (a) reagent storage and set-up, (b) sample preparation, (c) PCR reaction mix assembly and amplification, and (d) PCR product analysis. The following guidelines refer to these specific work areas.

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The respective rooms must be marked as related to the specific areas; on no account may equipment such as pipettors or reagents be removed from their respective work area or exchanged between work areas. Access to the individual work areas should follow a strict sequence, i.e., proceeding in only one direction, from the reagent storage and set-up area to the PCR product analysis area (access sequence). Previous experience has proven that ignorance, thoughtlessness, or carelessness in adhering to the said sequence can lead to the spreading of severe carryover contamination. Consequently, use of different laboratory coats (e.g., of different colors) is recommended in the individual work areas to identify directly a potential source of contamination. In addition, the space-specific coats must be left behind when workers leave the respective work area.

The procedures and the access sequence must be adhered to by all staff accessing the laboratory area, including the cleaning personnel, for improper cleaning procedures can be a major source of contaminants. In principle, the laboratory is cleaned from reagent storage and set-up in the direction of

the PCR product analysis area. Likewise, separate cleaning utensils should be available for each work area to prevent cross-contamination.

1.1. reagent storage and set-up

1.1.1. Operations.

The following operations are done in the reagent storage and set-up area: preparation of stock solutions, preparation of aliquoted solutions, and preparation of master mix solutions. Cleaning of the workplace has to be performed immediately after the termination of the work. Also, specific work areas must not be accessed if work was performed earlier in any of the other working areas, particularly in the PCR product analysis area.

1.1.2. Job description, work flow.

Delivery of stock reagents and material for sample preparation is best done directly to the reagent storage and set-up area. Delivery to the PCR analysis area should be avoided (see below). Vessels containing reaction mixtures should always be centrifuged briefly before opening. The required reagents are stored exclusively in this area and processed here into the stock solutions needed.

After the stock solutions have been checked for suitability, they should be divided into aliquots for storage and further use, to reduce the danger of contamination through frequent opening of reaction vessels and pipetting.

Vessels containing reaction mixtures should always be centrifuged briefly before freezing. In general, most solutions used for PCR are stored frozen. Frequent use of freeze/thaw cycles of master stock solutions to remove aliquots for individual reactions must be avoided; instead, freeze stock solutions in small aliquots. Because the required volume for these stock solutions is determined by the number of PCR reactions usually carried out in one assay run in the laboratory, no specific volume recommendations are given here. Aliquot stocks of suitable sizes are predispensed into microreaction vessels and are subsequently frozen for storage. Plastic containers such as TupperwareTM are ideal for keeping batches of frozen stocks.

In addition, the reagent storage and set-up working area can serve for setting up the master mix solutions, i.e., preparations containing all reaction components except the nucleic acids to be tested. The objective is to prepare aliquots of the master mix solutions into the appropriate reaction format for a given assay and to store them until required for use. The suitability and stability of reaction components, especially polymerase enzymes, are to be checked (e.g.) by pilot reactions, and the results of the evaluations have to be documented. For "hot-start" techniques (enzyme addition after a first denaturation step at high temperature), the polymerases are also omitted from the master mix.

1.1.3. Clothing.

Protective clothing should preferably feature close-fitting sleevebands and should close tightly at the front. Surgical gowns are ideally suited for this purpose and are usually available, at least in a hospital laboratory. Throughout the entire work, workers must wear gloves and should change them frequently. When leaving the area, workers must leave their protective clothing behind.

An additional safeguard against contamination of specimens is the use of disposable operating caps. These are worn only rarely in routine practice, but their use should be strongly considered, particularly in this work area, where a contamination may ruin whole sets of stock reagents and master mixes.

1.1.4. Equipment.

Pipetting by mouth is strongly prohibited. Pipettes and pipetting aids must be autoclavable. Electric microdispensers for aliquoting increase the pipetting precision and thus limit any aerosol formation caused by frequent up-and-down pipetting.

The working area is to be equipped with a -20 °C freezer, a 4 °C refrigerator, a precision balance, a pH meter, a chipped ice maker, and stationery. The surfaces of the workbenches should be able to withstand decontamination procedures with such chemicals as sodium hypochlorite (3). Ultraviolet irradiation of work surfaces is also effective. Given the critical importance of the distance and the energy of irradiation for decontamination, we use in our laboratories pull-down UV lights (254 nm wavelength) that can be adjusted to within ~60–90 cm (~2–3 ft.) above the workbenches after the work in the work area is finished. Also, because of the small size of a few hundred basepairs and the fact that dried aerosols are less susceptible than "wet" aerosols to UV damage, PCR fragments must be irradiated for extended periods—optimally, overnight (4)(5)(6). Finally, a logbook (or a similarly suitable means of documentation) for recording use of the laboratory space and equipment has to be provided.

1.1.5. Consumables.

Consumables include cleansing liquid and disposable tissues, disposable gloves, disposable caps (where used), autoclavable reagent vessels of various sizes, disposable weighing paper, reagents for nucleic acid preparation, and reagents for reactions (e.g., enzymes, buffers, dNTPs).

1.2. sample/template preparation and cdna synthesis

1.2.1. Operations.

The sample preparation work area serves as specimen storage area. In addition, the extraction of nucleic acids (RNA, DNA), their storage, and their delivery into prepared reaction vessels (see above) are performed in this area. Single-strand cDNA synthesis for RNA analyses is also done in this work area.

1.2.2. Job description, flow of work.

The reagents and consumables from this working area must not be taken into the PCR pipetting area, because they might be contaminated with unamplified nucleic acids. Also, leftover master mixes, enzymes, or reagents must not be transported back to the reagent storage and set-up area. Sample preparation must not be started after previous work in the PCR product analysis area (see below).

Good pipetting technique is instrumental for untroubled amplification procedures. Because contaminations from aerosol formation may occur, unnecessary moving around the laboratory should be avoided. Aerosol contaminations from the access area to the sample preparation area can be reduced by positive pressure conditions inside the laboratory. To avoid cross-contamination between samples, reaction vessels containing reaction mixes must be closed after addition of the test nucleic acids. For potentially infectious materials, established recommendations for handling and disinfection must be observed.

Used pipette tips must be disposed of exclusively in suitable decontamination containers, e.g., containing sodium hypochlorite solution. Laboratory bench tops must always be cleaned at the end of work, and any spillage of test material must be recorded.

Appropriate UV radiation of laboratory tables (254 nm wavelength, short distance to work bench surface) is suitable for decontamination. For safety reasons, we recommend a UV radiation source that can be switched on/off either from outside the room or by a timer. A pull-down UV tubular lamp mounted over the laboratory bench can be used to assure efficient irradiation of laboratory bench surfaces after work.

Use of suitable commercial systems based on liquid extraction/precipitation, adsorption of DNA to silica surfaces, or anion-exchange chromatography permits fast and trouble-free recovery of sufficient quantities of DNA or RNA from a variety of specimen types. Alternatively, the nucleic acids can be prepared according to accepted procedures with home-made reagents (e.g., (7)(8)(9)). The recovery of RNA is performed in a manner similar to DNA preparation. However, important differences are implied by the instability of the analyte and the omnipresence and stability of RNases. Moreover, several DNA extraction methods and commercial preparation kits require a RNase digestion of the DNA sample. If these reagents are used in DNA extraction and both types of nucleic acids are handled in the same area, particular care must be taken to guarantee RNase-free conditions and solutions for RNA work by using dedicated consumables and pipettes.

For various reasons, it is practical to carry out a cDNA synthesis immediately after the RNA preparation. The cDNA synthesis should be carried out in the sample preparation area to help avoid contamination. Being more stable than RNA, the storage of a first-strand cDNA is less critical. Also, performance of the first-strand synthesis in the "downstream" working area for PCR assembly entails storage of the samples, because their transport back into the sample preparation area is, by definition, prohibited. To achieve the flexibility required for this RT reaction, one or more thermoblocks should be set up in the sample preparation area.

The optimal temperatures for cDNA synthesis depend on the enzyme chosen. One-step methods are preferable; i.e., use of heat-stable polymerases with RT activity under PCR buffer conditions (example 1, below) is safer than methods that follow cDNA synthesis by requiring opening of the reaction vessels for purposes of buffer adjustment (example 2, below) or polymerase addition (example 3, below). When using enzymes that possess both RT and DNA polymerase activity, one should assess the RNA dependence of the assay separately in the examination of intron-free genes as well as possible interference from processed pseudogenes. Examples of assay strategies currently in use are:

- 1) Addition of a polymerase with RT activity (e.g., Retrotherm from Epicentre Technology) → cDNA synthesis → no opening of vessels → PCR
- 2) Addition of a polymerase with RT activity (e.g., rTth from Perkin-Elmer) → cDNA synthesis → opening of vessels → adjustment of buffer to PCR conditions (here, chelate buffer) → PCR

3) Addition of RT (e.g., AMV-RT from Pharmacia) → cDNA synthesis → opening of vessels → addition of polymerase and PCR components → PCR

The cDNA copies of the test material are kept in the sample preparation area. PCR amplification from specimens is not allowed in this area.

1.2.3. Clothing.

Marked laboratory clothing as described above; frequent change of gloves.

1.2.4. Equipment.

Workbench with hood and UV radiator (see *Sections 1.1.4* and *3.2.2*) and positive-displacement pipettors or regular pipettors in conjunction with aerosol-proof disposable pipette tips. The pipettors should be autoclavable. Dispensers, freezer (-20 °C and -80 °C), refrigerator (4 °C). Additional equipment includes a vortex-type mixer and a waterbath or heating block. A logbook (or similarly suited means of documentation) must be available.

For RNA work, two additional pieces of equipment are recommended:

1) A cooling microcentrifuge (e.g., from Eppendorf) or centrifugation in a cold cabinet is preferred for RNA work for the following reasons: First, RNA preparations are best done on ice because of the low stability of the analyte and, depending on the specimen material, because the precipitation of RNAs is a usual step in preparation protocols (8). Second, when stored for extended periods of time, RNA is usually kept as an ethanol precipitate, also for reasons of stability, and requires centrifugation before use in RT reactions. Centrifugation in the cold minimizes the risk of degradation while handling RNA.

2) Depending on the method used and the specimen investigated, shearing of the high-molecular-mass DNA, commonly done by passing the samples through a syringe needle (8), may be necessary to reduce the viscosity of the material. However, this bears a substantial risk of contaminating the work area with unamplified nucleic acids, is hazardous with respect to handling, and is also impractical for routine purposes. Instead, high-molecular-mass DNA may be degraded by using a suitable ultrasonic water bath, obviating the need to open the sample tubes.

1.2.5. Consumables.

Cleansing liquids and disposable tissues, aerosol-tight pipette tips, autoclavable reagent vessels, gloves, disposable caps, reagents for preparation of samples (prepared in reagent set-up area).

1.3. pcr reaction mix assembly

1.3.1. Operations.

Dispensing of sample material (from sample preparation area, see *Section 1.2*) and master mix solutions (from reagent storage and set-up area, see *Section 1.1*) into the reaction mixtures as well as the amplification reactions is performed exclusively in this working area. In nested PCR assays, it is usually necessary to open the vessels after the first round of PCR. Consequently, nested PCR possesses a substantially higher contamination risk and therefore demands particularly strict attention. The setting aside and installation of this working area should be obligatory for all nested PCR applications.

1.3.2. Job description, flow of work.

Amplification of DNA fragments or of first-strand cDNA fragments is to be done exclusively in this working area. There should be no access from this section to any "upstream" working areas. Working under conditions of reduced atmospheric pressure is advisable to impede any leaking of aerosol contaminants from this area.

To avoid contaminations by aerosol formation, work should be set up beforehand, and any moving around the laboratory should be kept to a minimum. Pipetting should be performed in a hood. The opening of predispensed reaction mixes must be performed with great care, particularly between nested PCR steps. A good procedure is to briefly centrifuge all liquids in the reaction tubes before opening. Very small centrifuges (e.g., PicoFugeTM) from Stratagene) are ideally suited for this purpose; they take up very little bench space, are easily operated with one hand, and fit into most work cabinets. Moisture barriers, such as paraffin wax or light mineral oil, provide additional protection against contamination. However, one must be aware that mineral oil itself can be the cause of persistent contaminations. Used pipettes must be disposed of, preferably in containers filled with decontamination liquids (see *Section 3.2.2*). We also recommend analyzing samples in duplicates or amplifying two different DNA sequences.

Commercially available wax pellets can be used for hot-start assays. Alternatively, a chemically denatured (and, in this state, inactive) DNA polymerase (TaqGOLDTM; Perkin-Elmer) has recently been introduced. Upon prolonged exposure to high temperatures near 94 °C, the enzyme will reactivate, thereby enabling an elegant hot start of PCR. For nested PCR, increased attention must be given to possible splashes when opening vessels. A brief centrifugation before opening reaction vessels is especially important. For pipetting purposes, reaction vessels should be opened under a safety hood.

Laboratory bench surfaces must always be cleaned and decontaminated after finishing work and at the end of the workday. Again, any spilling of solution is to be recorded.

1.3.3. Clothing.

Marked laboratory clothing as described above, and wearing of gloves and caps. In this area, gloves should be changed frequently. Clothes must be left behind when leaving the room.

1.3.4. Equipment.

Workbench with hood and UV radiator (see *Sections 1.1.4* and *3.2.2*), autoclavable pipettors, thermocycler, waterbath, stationery, electric micropipettor, and one or two PicoFuges (see above).

1.3.5. Consumables.

Cleansing liquids and disposable tissues, aerosol-tight pipette tips, autoclavable reagent vessels, gloves, caps, and reagents for assay preparation (produced in pre-PCR area).

1.4. per product analysis**1.4.1. Operations.**

Operations depend on the sensitivity (detection limit) of the method for detection of PCR fragments.

1.4.2. Job description, flow of work.

For detection of amplified PCR fragments, a variety of methods are available, including those listed here. The method to use depends on the analytical problem to be solved.

Agarose gel electrophoresis

Polyacrylamide gel electrophoresis

Nondenaturing

Denaturing gradient

Hybridization method (radioactive, nonradioactive)

Solid phase

Dot-blot

Southern transfer

In solution

ELISA

Sequencing methods (radioactive, nonradioactive)

Other methods

The analysis of amplification products inevitably leads to a contamination of this area by PCR fragments. In contrast to the other PCR working areas, the following safety aspects have to be considered to protect the laboratory personnel in this area:

- 1) Common use is made of mutagenic and toxic substances, e.g., ethidium bromide, acrylamide, formaldehyde, or radioisotopes (the pertinent radiation protection recommendations are applicable, including wearing of personal dosimeters, where necessary).
- 2) When handling fragments previously amplified from genes with an oncogenic potential, appropriate protective measures should be considered. Laboratory staff should be informed accordingly.

1.4.3. Clothing.

Normal laboratory and protective clothing.

1.4.4. Equipment.

Depends on method used.

1.4.5. Consumables.

Depend on method used.

► 2. Preanalytical Aspects

The techniques used in the amplification of genetic information require special emphasis and a clear definition of the preanalytical steps, especially when these techniques are applied to diagnosis. On the one hand, nucleic acids (especially RNA) can easily be destroyed through ubiquitous nucleases; on the other, the extremely high sensitivity of PCR necessitates the adoption of special precautions to protect the sample against contamination by unrelated nucleic acids (e.g., from laboratory personnel).

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The quality of the test results is influenced to a high degree by factors that affect sampling and dispatch of samples. Aspects of sampling, sample storage before dispatch, and dispatch itself must be seen as important elements of the preanalytical stage. Minor mistakes in connection with preanalytical steps that are not standardized may have severe consequences for the test result. In the individual case it will be difficult, if not impossible, for the laboratory to reconstruct any such mistakes made by the sender.

In the following sections, sample preparation is differentiated from the preanalytical step and is defined as part of the analytical stage, because the choice of method depends on the intended application, and the sensitivity of the assay will depend on the preparation procedure.

2.1. specimens

In principle, PCR analysis can be applied to a wide variety of materials, including whole blood or bone marrow containing an anticoagulant such as EDTA or citrate, serum or plasma, dried blood (filter-paper cards), buffy coat, sputum, mouthwash, bronchial lavage, cerebrospinal fluid, urine, stool, biopsy material, cell cultures, fixed tissue, embedded tissue, tissue sections, and so forth. Because heparin inhibits PCR reactions, specific precautions have to be considered when the use of heparinized material is intended (see *Section 2.2*). Depending on the test material, pretreatment of the sample may be necessary before stabilization, e.g., liquefaction of sputum with hyaluronidase.

2.2. sampling

Sampling is best done in closed, disposable sampling systems, as is customary with other clinical test material. New disposable plasticware can be considered nuclease-free and used without further pretreatment. Where nonclosed sampling systems are used, e.g., for urine, secretions, stool, or bone marrow, or when particulate specimens like hair are obtained, special attention must be given to protecting the sample against contamination with (e.g.) hair, epidermal scale, or sputum from the sampler. At the very least, disposable gloves must be worn.

With respect to reusable general glassware, the following should be considered: Glassware should be autoclaved or even better, heat-sterilized, because (a) sterile glass equipment is not necessarily free of

contaminating DNA, and (b) glassware is often a source of highly resistant RNA-degrading enzymes, a major source of which is the hands of the investigator. RNases can be permanently inactivated through high temperatures, e.g., by baking at 250 °C for 4 h or more (9).

In blood and bone marrow specimens, clotting must be inhibited. EDTA and citrate are commonly used and are the preferred inhibitors. The citrate-containing specimen collection systems (also used for routine blood coagulation tests) will dilute the specimen by 10%. In contrast, heparin (routinely 14.3 IU/mL of whole blood) reportedly inhibits amplification in concentrations as low as 0.05 IU per reaction volume (10). For heparinized specimens the following must be taken into consideration:

- 1) For simple PCR tests not requiring high sensitivity, dilution of the prepared nucleic acids is ordinarily sufficient to overcome the inhibition. If heparinized material has to be used and a more sensitive DNA PCR is required, nucleated cells should be isolated first and washed repeatedly in physiological buffers before further processing.
- 2) Where highly sensitive RT-PCR methods are required, additional measures are necessary to overcome heparin inhibition. Methods shown to fail at this are boiling, Sephadex chromatography, pH shifts with subsequent gel filtration, repeated ethanol precipitations, and treatment with protamine sulfate. Although treatment with heparinase restores the amplification (11)(12), this enzymatic purification step is costly. In addition, RNA may be degraded during enzyme incubation by traces of RNase still present in the sample or by heparinase preparations contaminated with RNase.
- 3) As demonstrated recently, lithium chloride can separate heparin from RNA, thus reversing the inhibition. This method, which reliably restores amplification from heparinized blood samples, is easily incorporated into a routine RNA preparation procedure without additional effort (13).

2.3. sample fractionation

When necessary, target cells can be enriched before sample stabilization and dispatch. Enrichment is useful when specimens contain low numbers of cells (e.g., urine, ascites, secretions, excretions). The sample should be centrifuged at low speed before stabilization.

In specimens containing high numbers of nonnucleated cells, e.g., blood or red bone marrow, stabilization may be preceded by a selective lysis of erythrocytes and followed by recovery of the nucleated cells through centrifugation at low speed. Another common method of sample fractionating is Ficoll density-gradient centrifugation. When performed at the site of specimen sampling, problems may arise from poor standardization, variable recovery of target cells, and danger of specimen contamination. Problems with standardization of Ficoll gradient enrichment of nucleated cell populations may be circumvented in the future by using combined sampling/fractionating systems, e.g., the Vacutainer Tube CPT tubes^(TM) (Becton Dickinson).

2.4. sample stabilization

Stabilization of test material is essential because nucleic acids degrade rapidly and is especially important when RNA has to be analyzed. Instant inactivation of DNases and RNases is reliably achieved

by chaotropic substances (especially guanidinium isothiocyanate, GITC). GITC has been increasingly used in concentrations of 4 mol/L as originally described (8). Organic solvents, e.g., phenol, may be added in parallel. Extraction systems based on these additives are now commercially available, e.g., RNAzol, Trizol. However, the limited stability of reducing agents (β -mercaptoethanol or dithiothreitol) and their requirement for sample stability need to be considered. Therefore, the user must be aware that batches of ready-to-use extraction solutions have a limited shelf life because of instability of some of their individual components, e.g., β -mercaptoethanol. Moreover, the handling of organic solvents is hazardous to human health and due care must be taken during handling.

At the site of specimen collection, original or enriched material is lysed by addition to reagent tubes containing GITC. The appropriate concentration leading to an irreversible denaturation of RNases has recently been determined to be 5 mol/L. Use of GITC concentrations <4 mol/L leads to very rapid RNA degradation (14). After proper stabilization, the material usually need not be cooled before mailing for analysis. At temperatures cooler than room temperature, GITC will crystallize, thereafter requiring complete thawing before addition of the specimen. Depending on the abundance of the RNA target to be amplified, chelating agents, e.g., citrate (combined with low temperatures), may be appropriate to inhibit RNases, given that the enzyme activity of the RNases depends on free divalent cations (9). In our experience, GITC is preferable where maximum sensitivity is required and where delays in transport to the laboratory cannot be excluded. In any case, the suitability of a stabilization system should be documented with respect to the sensitivity of the subsequent RT/amplification reactions.

For extraction of DNA from leukocytes, blood containing EDTA as anticoagulant requires no special stabilization; nonetheless, samples should be dispatched without delay to the laboratory.

2.5. sample dispatch

Samples stabilized appropriately may be dispatched by regular mail at ambient temperatures. This applies to EDTA-containing whole blood for DNA preparation and GITC-stabilized specimens for RNA recovery. Cooling is not necessary but depending on the application, prolonged storage at room temperature will result in a critical loss in sensitivity (14). In general, samples should be dispatched in breakproof containers. RNA targets to be investigated in nonstabilized samples must be shockfrozen and then dispatched in solid CO₂. Samples that reach the laboratory in the state of thawing should be invariably rejected.

2.6. sample storage

Specimens for DNA analysis should be stored in buffers of 10 mmol/L Tris, 1 mmol/L EDTA (pH 7.5–8.0), at 4 °C. Specimens for RNA analysis should be kept in buffered solution preferably at -80 °C or in liquid nitrogen. Equally suitable is storage as an ethanol precipitate at -20 °C. GITC-stabilized RNA samples may be stored for ~7 days at room temperature. In cases of longer storage, less-sensitive limits of RNA detection have been observed. Such findings must be taken into account when only a few viruses or cells are to be detected.

► 3. Factors Interfering with Analytical Procedures

3.1. preparation of specimens

RNA or DNA (e.g., from human cells or viruses) may be isolated from a multitude of different specimens (see *Section 2.1*). For DNA analysis, no special measures are usually necessary if sample transport to the laboratory and DNA preparation are performed without delay. When RNA has to be analyzed, degradation of the analyte may be prevented through stabilization as described above.

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3.1.1. Interferences related to DNA preparation.

DNA preparations of inferior quality are often characterized by incomplete removal of inhibitors, either from the sample itself (e.g., heme, its precursors, or degradation products (7)) or introduced during inadequate sampling (e.g., heparin [10]). When phenol is not completely removed, the subsequent enzymatic steps (e.g., PCR, restriction digest) may be inhibited (15). In this case, the DNA should be taken up in a larger volume of buffer before phenol extraction, chloroform extraction, and repeated precipitation. Although usually not required, traces of chloroform may be removed by ether extraction (9). When starting with very small amounts of sample material (e.g., Guthrie filter cards, tissue sections), losses in nucleic acid may occur during preparation. Such losses can be minimized by adding carrier tRNA (Gibco BRL) or glycogen (Boehringer Mannheim) during the precipitation steps (9). Other reagents of suitable quality (molecular biology-grade) can be purchased from several commercial sources. Where used according to manufacturer's instructions, such reagents do not interfere with the subsequent enzymatic reactions.

Long-term storage of DNA should be done exclusively in buffered solutions. Tris-EDTA buffer (10 and 1 mmol/L, respectively, pH 8.0) is well suited. In water, autocatalytic processes through depurination can result in a completely degraded DNA after only a few weeks. DNA appropriately prepared may be stored in buffered solutions at 4 °C for years without any large losses in quality or quantity.

3.1.2. Interferences related to RNA preparation.

The sensitive detection of intact RNA is frequently compromised by inhibitors that have not been eliminated completely, e.g., heme and heparin, as described above. RNA degradation presents a major problem. Common causes for a failure of RT-PCR assays are insufficient sample stabilization before sample dispatch and RNase contamination of reagents for preparation. For the former, the logistics of the preanalytical phase need to be checked; if there is evidence for RNA degradation, the specimen should be rejected by the laboratory, which should order a new specimen and give detailed instructions to the sender regarding the proper procedure. For the latter, use of commercial preparation kits, quality-checked by manufacturers, is recommended for routine tests.

In addition, RNA may be lost during precipitation or long-term storage of prepared RNA. A loss, especially of polyA-mRNA, may be prevented through coprecipitation with glycogen (10 g/L in diethylpyrocarbonate-treated water), which gives a clearly recognizable visible precipitate. Because RNA is unstable in water, long-term storage as an ethanol precipitate at -20 °C or -80 °C is recommended.

3.2. reverse transcription and amplification of target sequences

3.2.1. *Interferences with cDNA synthesis.*

cDNA synthesis is the first enzymatic step in RT-PCR. The cDNA generated as the reverse complement from the target mRNA then serves as the template for the subsequent amplifications.

cDNA synthesis is convenient when the amplification of single exons from DNA is not feasible, when no information is available regarding genomic sequences or gene organization, or when disease-associated or disease-specific transcripts are to be detected. In addition, analysis of a few cDNA fragments instead of many genomic fragments may be advantageous. For prospective studies, it may be safer to translate mRNA into cDNA to achieve better conditions for long-term storage.

The following factors commonly affect the efficiency of cDNA synthesis:

- 1) Reduction or complete absence of RT activity. Inadequate cDNA synthesis caused by low enzyme quality, decomposed reagents, or pipetting errors must be excluded.
- 2) Inhibitors of the RT or of the heat-stable polymerase (e.g., phenol, heme). These are to be suspected when no amplification can be achieved even though the RNA appears to be intact. Amplification of a sufficiently expressed housekeeping gene (e.g., β_2 -microglobulin, glyceraldehyde-3-phosphate dehydrogenase) may serve as control if the sample, and thus the inhibitor, is appropriately prediluted.
- 3) Degradation of the RNA (which can be checked through appropriate controls during or after preparation). A degraded mRNA may be assumed when the ribosomal RNA populations 28S, 18S, and 5S are no longer clearly defined, or are possibly completely absent. A quick check with electrophoresis in a nondenaturing, ethidium bromide-prestained agarose gel is usually sufficient. This simple test requires ~1 μ g of total RNA for reasons of detectability; thus it is not suited for all methods. A more thorough assessment of whether the RNA species is intact requires agarose gel electrophoresis under denaturing conditions.
- 4) Contamination of RNA with genomic DNA. Primers for the amplification of cDNA fragments usually can be positioned to hybridize in different exons, which will warrant RNA-dependent amplification. Where this is the case, PCR products from contaminating non-mRNA nucleic acids, should they occur, are larger than those obtained from cDNA. Where this design is not possible, e.g., with intron-free genes or intron-free pseudogenes, the RNA preparation is treated with RNase-free DNase. For such targets, an amplification without a preceding RT step must be performed to control sufficiency of this step. This control is not recommended when one is using enzymes that possess both RT and DNA polymerase activity (e.g., rTth).
- 5) Residual DNase activity. If RNA samples are treated with RNase-free DNase before first-strand cDNA synthesis, care must be taken to remove the DNase activity before the reverse transcription step. Usually, 10 min at 65 °C is sufficient and will also aid in breaking up secondary structures in the RNA template that can interfere with efficient reverse transcription (9). The RNA sample can be extracted with organic solvents/precipitation, but this may result in loss of material or inhibition of the subsequent

enzyme step because of trace amounts of phenol in the reverse transcription reaction.

6) Insufficient priming of mRNA in the first-strand cDNA synthesis attributable to, e.g., the type of priming. Three different methods for the initiation of first-strand cDNA synthesis exist: oligo(dT) priming, random priming, and transcript-specific priming. *Oligo(dT) priming* starts the reverse complementary first-strand cDNA synthesis at the 3'-end of the mRNA. An unfavorable secondary structure of the mRNA or a long mRNA sequence can lead to the first-strand cDNA being not fully reverse-transcribed towards the 5' end of the mRNA. Consequently, amplification is not successful in the subsequent PCR amplification, even if the mRNA has been successfully prepared from the specimen. *Random priming* starts cDNA synthesis from short primers with random sequences (hexamers or octamers). The cDNA synthesis initiates at random sites and, theoretically, will thus cover all RNA species present in the sample. The average length of the cDNA depends on the molar ratio of random primers and mRNA, with a high excess of random primers favoring short DNA fragments. If this is the case, subsequent PCR amplification may be compromised. For *target-specific mRNA priming*, the suitability of the respective 3' primer for cDNA synthesis has to be established.

3.2.2. Factors affecting PCR.

Various factors can lead to false-negative or false-positive results in PCR, e.g., inhibitors or the absence of enzyme activity (see above), inappropriate annealing temperature, suboptimal magnesium concentration, or contamination of patients' samples or reagents, each of which will be discussed separately.

Of particular importance is the selection of the proper primer pairs. Usually, commercially available primers are high in quality, especially when further purified by the vendor, e.g., by HPLC chromatography. Today, numerous 5'-end modifications can be ordered, allowing labeling of the oligonucleotides with fluorochromes, biotin, amino linkers, and others. If one wants to design a test "from scratch," however, much thought should be given to the initial primer design. Several computer programs—either part of a regular DNA analysis software package or a stand-alone program that can be ordered—can help avoid unfavorable secondary structures, predict the occurrence of primer dimer formation, allow approximations of optimal annealing conditions, and so forth. (Evaluation of the algorithms underlying primer calculation or program features is beyond the scope of this document.) Once a primer is identified, the investigator should "run" it through the genetic database to check for cross-hybridization. This can be done conveniently through the Internet by using the BLAST programs displayed in the National Center for Biotechnology Information homepage (<http://www.NCBI.NIH.NLM.gov>). Certainly, however, the performance of a chosen primer pair identified through a software still requires careful evaluation with respect to specificity and amplification efficiency during the phase of test establishment.

Inhibitors of PCR are to be avoided in the preanalytical phase and have to be eliminated during sample processing within the laboratory.

The annealing temperature is dependent on the sequence, i.e., length and base composition, of the amplification primers. In assessing the optimal temperature, one may apply the Wallace rule as a rule of

thumb; i.e., the melting temperature of an A–T bond is in the range of 2 °C and that of a G–C bond is in the range of 4 °C. As with the use of computer programs (see above), the optimal primer performance always has to be investigated systematically by suitable pilot experiments.

The optimal magnesium concentration has to be established by appropriate titration experiments. The optimal concentration window may be very narrow. As a rule of thumb, one should initially start by using the standard concentration recommended by the manufacturer of the enzyme. Because the free magnesium ion concentration determines the efficacy of the enzymatic nucleic acid polymerization, the nucleic acid content or the individual concentrations of other magnesium-binding polyanions in the preparation will influence the amplification result. An optimal concentration of magnesium should result in a maximum yield of PCR, and no unspecific bands should be detectable by agarose gel electrophoresis.

Uniform temperature transition is an important aspect for a successful amplification. For example, the wall thickness, the material making up the reaction vessels, and a good fit of the reaction vessels into the thermoblock well are essential.

The homogeneity of heat conduction in the reaction block is of crucial importance. The heat performance of the cycler and the uniformity of heat conduction in the heating block must be controlled regularly to avoid false-negative results.

During test evaluation or in case of uncertain interpretation, the identity of the PCR product must be certified by proper means, i.e., by restriction fragment polymorphism, hybridization, or, ideally, by DNA sequencing of the purified PCR fragment.

3.3. contaminations

For practical reasons, the following types of contamination are distinguished here: contamination with PCR fragments (product contamination); contamination with native, genomic DNA; reagent contamination (stock solutions or working solutions); and cross-contamination (e.g., spreading of aerosols from a positive sample into an originally negative sample).

For all amplification techniques, greatest attention is directed towards the prevention of contamination, because locating its source around the laboratory is time-consuming and tedious. Once a contamination has occurred, testing has to cease, until the source is identified. Without exception, test results must be rejected, even if only one of the accompanying contamination controls reveals contaminating PCR fragments.

3.3.1. Contamination sources in the preanalytical phase.

Nucleic acids not originating from the patient may contaminate the test material during the preanalytical phase.

3.3.2. Contamination sources in the analytical phase.

As a general rule, contamination of the sample may occur anywhere during the different steps of the analytical phase. Accordingly, any component of the reaction mixture and any piece of laboratory

equipment coming into contact with the reaction during nucleic acid preparation and reaction set-up is suspicious as a source. Examples include: contaminated reagents (e.g., bovine serum albumin, gelatin, or mineral oil); commercially available enzyme preparations (16); consumables (e.g., reaction vessels, pipet tips); and laboratory equipment (e.g., pipettors, centrifuges).

One origin of contamination is cross-contamination with unamplified DNA during the simultaneous preparation of many specimens. Most contaminations, however, will consist of the specific PCR fragments generated during previous amplifications.

A contamination of reagents, consumables, or laboratory equipment used in the first three work areas (see *Section 1*) may indicate improper laboratory procedures. In contrast, contamination of the product analysis area is inevitable when PCR products are pipetted. Great care must be taken to identify this sort of contamination. Specifically, one main source is the microdrops that cross-contaminate samples, e.g., when these are loaded onto agarose gels or onto dot-blot equipment. Whether or not such contaminations are detected and thus influence the test result depends on the detection limit of the method used to detect the PCR fragments. For example, a contamination not detected in ethidium bromide-stained agarose gels may very well become apparent if the method is changed to a sensitive hybridization protocol. Multiple analyses of the same sample, run at different positions on a gel or in different runs, will help to exclude such cross-contaminations as the cause.

3.3.3. Avoidance of contamination.

As a general rule, prevention rather than removal must be given highest priority. Individual aspects have already been dealt with earlier in the context of dividing the working areas and the logistical separation of assay steps.

Several methods exist to destroy amplification products generated in previous assay runs before they can evolve into a detectable contamination. As a general safeguard, for example, amplification reactions can be performed with reaction mixes, in which dTTP is partially replaced by dUTP, thereby generating uracil-containing specific amplification fragments. A preamplification digestion of reaction mixes with uracil-*N*-glycosylase will therefore destroy contaminations carried over from previous assays (17). An alternative "post-PCR sterilization" is photochemical generation of DNA adducts by isopsoralen compounds in the presence of longwave UV light (18)(19). These also prevent contamination, because the DNA adducts are refractory to amplification but do not interfere with post-PCR hybridization procedures.

However, the aforementioned methods are not generally recommended and should be viewed with caution, because they can generate a false feeling of safety. In particular, these measures will not prevent contaminations from foreign, nonamplified native DNA.

3.3.4. Decontamination measures.

Efficient decontamination at regular intervals after termination of work is mandatory. A combination of various methods promises the best results. Decontamination measures include, but are not limited to: chemical cleaning of surfaces with 100 mL/L sodium hypochlorite (3); permanent UV radiation (254

nm) of laboratory benches and other surfaces after use (4); autoclaving of laboratory equipment, e.g., pipettors; and flaming of laboratory equipment, where possible.

3.4. analysis of amplification products

Various techniques can be used to evaluate amplification products, including electrophoresis, restriction digestion, blotting, hybridization, sequencing, and mass spectrometry, each of which may be subject to specific disturbances. Assessing the specificity of a PCR only on the basis of the product's fragment length determined by simple agarose gel electrophoresis is to be reserved for well-established assays.

3.4.1. Interferences in electrophoresis.

In general, two different types of electrophoretic separation techniques are used in molecular biological diagnosis: submarine agarose gel electrophoresis and polyacrylamide gel electrophoresis. Both techniques can be applied under either denaturing or nondenaturing conditions. As with the electrophoretic characterization of proteins, the following factors affect the electrophoretic characterization of PCR fragments:

- 1) False gel concentrations, ionic strength, or pH. A careful control of reagents or the use of commercially available ready-to-use reagents checked for their quality by the manufacturer is suggested.
- 2) Imperfect sample preparation. High salt content of the sample (e.g.) can affect the electrophoretic mobility and the band pattern of the DNA fragments and may lead to incorrect estimation of the fragment size.
- 3) Overstaining DNA products or using insufficiently sensitive stain. The intercalating fluorescent stain ethidium bromide can differ in sensitivity between manufacturers or lot-to-lot. The right stain concentration is easily established through use of amplification products as controls. (*Note:* Intercalating DNA stains are strong carcinogens; therefore, contaminated buffers must be properly disposed of.)

3.4.2. Interferences related to restriction digestion.

Restriction digestion may precede a PCR reaction so as to increase the specificity of the reaction (e.g., cutting a processed pseudogene in a RT-PCR) or to detect the methylation of target sequences (e.g., X-inactivation). The main application of restriction digestion, however, is either to verify the specificity of a PCR product or to detect gene mutations via defined restriction fragment lengths (restriction fragment length polymorphism, RFLP). In comparison with restriction of unamplified DNA, the following must be considered when amplification products are to be digested:

Usually, the unit definition, as given by the manufacturer, is the basis for assessing the enzyme concentration required for the restriction digestion. One unit is defined as the quantity of enzyme that completely cuts 1 µg of DNA at a defined temperature within 1 h. The abundance of a restriction site in the test DNA—assuming a uniform base distribution—statistically depends on the length of the recognition sequence of the enzyme: e.g., for a recognition sequence of 4 bases, 1 site/4 = 256 bases; for a recognition sequence of 6 bases, 1 site/4 = 4096 bases. Obviously, this does not apply to a PCR product, because the "cutting site density" in the digestion reaction is very high compared with the naturally occurring sequences, thereby rendering the mass-related enzyme concentration a limiting

factor. Accordingly, the unit definition is not valid for amplification products, and the amount of enzyme necessary for digestion must be determined empirically. The manufacturers provide information on the characteristics of restriction enzymes, e.g., inhibition by glycerol or star activity, and the related quantity per volume to be used.

Further major complications are insufficient or absent activity of the restriction endonuclease, possibly caused by the presence of certain compounds in the amplification reaction mix. For example, the salt conditions required for cutting may be incompatible with the salt conditions used for amplification. If in doubt, use of organic solvent extraction and subsequent precipitation most often will solve a cutting problem.

Another cause for insufficient or absent digestion is decreased activity of these temperature-sensitive enzymes.

The assessment of a quantitative restriction digestion must be assured through appropriate internal and external controls, because a partial digestion can entail misinterpretations of the banding pattern.

3.4.3. Interferences related to nucleic acid transfer (blotting).

There are essentially two reasons for the transfer and immobilization of DNA from a gel to a solid matrix (e.g., nylon membrane, nitrocellulose), namely, to increase the specificity of detection of a PCR product and to decrease the detection limit relative to simple staining with fluorescent dyes.

Even in cases of a professional performance of blotting, the presence of membrane regions in which the binding of nucleic acids is reduced or even absent may compromise results. Avoidance of membrane artifacts is a demand addressed to manufacturers.

3.4.4. Interferences related to hybridization.

Inadequate hybridization results can be caused by inappropriate gene probes, inappropriate labeling methods, inadequate labeling of probes, or inappropriate hybridization or washing methods.

The gene probes most commonly used are: DNA fragments, synthetic oligonucleotides, and in vitro transcripts (antisense RNA probes). Probe production or labeling is mostly done with commercially available enzymes that are suitable for the respective labeling strategy. The enzymes most frequently used are: Klenow polymerase; T7 DNA polymerase; thermostable enzymes, e.g., Taq polymerase; T₄ polynucleotide kinase; terminal deoxy nucleotidyltransferase (tdT); T7 RNA polymerase; and SP6 RNA polymerase.

The efficiency of the labeling of a gene probe must be determined after each labeling reaction, because the sensitivity of the hybridization and thus the possibility of false-negative findings depends, among other factors, on the specific activity of the hybridization solution.

With radioactive labeling, the activity of a fraction of the labeled preparation is measured in a β -scintillation counter. The specific activity of the gene probe (counts/minute per microgram of probe) is

then adjusted according to the total incorporated counts and the amount of probe used for labeling. For example, radioactively labeling a synthetic oligonucleotide by using T_4 polynucleotide kinase with [γ P] ATP typically generates a specific activity of 1×10 to 5×10 counts/min per microgram. With nonradioactive labeling, the specific activity can be determined via a dilution series with subsequent detection in dot-blot. With both labeling methods, one must make sure that none of the measured signal is from non-incorporated label, e.g., radionuclide, fluorescence label, or a biotin-derivatized base.

To ensure reproducibility, the specific activity at time of labeling, the age of the probe at time of use, and the quantity of probe used in the hybridization preparation all must be recorded.

Hybridization conditions must always be ascertained empirically in the laboratory when a particular probe is first introduced into the test program. Accepted conditions as well as melting temperatures computed by oligonucleotide software programs can usually be used for a first approximation during the evaluation process. We recommend strict adherence to a fixed hybridization program once it has been established.

Both a temperature too low or an ionic strength too high will reduce the stringency of hybridization and may negatively affect the specificity of the detected signal. In contrast, raising the temperature, decreasing the ionic strength, or both, will increase stringency. Thus, a tight control of temperature and of reagents is the prerequisite to avoiding false-positive as well as false-negative results. Note that temperature and ionic strength should never be changed at the same time.

3.4.5. Interferences related to sequencing.

DNA sequencing, the most accurate method of determining the primary base composition of a DNA fragment, is imperative when controlling the authenticity of PCR products and has the highest analytical specificity for detection of point mutations in PCR products. Sequencing should be performed directly from the amplification reaction after PCR.

Because of absent 3' exonuclease activity, Taq polymerase and some other heat-stable polymerases cannot correct primary incorporation errors (absent proof-reading activity). According the amplification conditions used, misincorporations of nucleotides into the growing DNA chain (Taq polymerase errors) will occur with various frequencies. A considerable fraction of the DNA molecules generated will differ from the original sequence as a result of these misincorporations. For Taq polymerase, the magnitude of these substitutions ranges from ~2 to 4 nucleotides per 10 000 synthesized bases. Other enzymes will have different error rates.

A misincorporation randomly introduces a mutation into the sequence that will subsequently be amplified during the remaining PCR cycles. Still, at the end of the amplification run, errors will be underrepresented in comparison with the authentic sequence at any position in the fragment, unless a very low target number was amplified in the first place and the misincorporation occurred within the first cycles. Because the risk for the latter will increase with the length of the DNA segment amplified, the amplification of shorter fragments should be favored. During direct sequencing of the fragments in the PCR reaction, the authentic signal will be the highest at any given position in the amplified fragment.

In contrast, when the amplification products are cloned into plasmids and the recombinant clones (harboring individual PCR amplicons) are subsequently sequenced, incorrect base sequences will result as a consequence of the misincorporations "singled out" by the cloning process. The importance of this problem is aggravated for assays that are designed for the amplification of a very low number of target nucleic acid molecules, the use of a high number of cycles, or both.

Enzymes with low error rates, so-called high-fidelity (or proofreading) polymerases, are commercially available. They should be used for amplification where the cloning of the PCR product is desirable and the probability of isolating the authentic gene segment has to be guaranteed. However, these enzymes are generally more expensive and do not possess the same processivity as the nonproofreading enzymes.

In summary, only the sequence analysis determined directly from the uncloned PCR products (direct sequencing) can determine the correct DNA sequence and is the method of choice for detecting mutations in a heterozygous state.

Poor template quality, defective sequencing reagents, or poor primer specificities are easily detectable by the ambiguous or absent sequence ladder. Where nonradioactive sequencing methods and software-supported base-calling is used, the software design or settings should be appropriate to allow the identification of samples with heterozygous genotypes, i.e., allelic polymorphisms or point mutations. Towards this end, sequencing reactions must be optimized for low background signals. Results that are not unequivocally clear must not be used for interpretation; instead, the procedure needs to be repeated. While it is a good idea for increased confidence to routinely sequence both strands of the amplified fragment, sequencing of the opposite strand is mandatory when problems with the base-calling persist.

► 4. Quality Controls

As mentioned above, a variety of controls should be implemented to assess the quality of single steps during analysis, e.g., for RNA integrity, its suitability for amplification, sensitivity. With increasing automation and standardization, the number of controls to be performed has to be reassessed. As in conventional clinical chemistry, internal and external quality control can be distinguished.

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4.1. internal quality control

A considerable number of controls must be carried out in the laboratory to monitor the complex steps during DNA or RNA analysis. Such controls are mandatory for arriving at a conclusive interpretation of findings and serve to avoid false-positive and false-negative results. The production of reagents, the preparation of test material, PCR preparation itself, and each of these steps in its own right require thorough control because of the high analytical sensitivity of the PCR; the same applies, with appropriate alteration of details, to other enzymatic steps that may precede or follow amplification reactions.

4.1.1. Controls related to preparation of the test material.

For control of DNA preparation, agarose gel electrophoresis is most commonly used. The average length of the DNA is ~100 kb in routine manual preparation methods. In DNA preparation kits suitable for PCR, the average range is between 30 and 40 kb. However, clearly degraded DNA also generates strong fluorescent signals after electrophoretic separation and staining with ethidium bromide in a lower-molecular-mass range between 1 and 10 kb. Through digestion of the DNA with a methylation-insensitive restriction endonuclease, e.g., *EcoRI*, followed by electrophoretic separation, one can control for inhibitors of enzymatic activities (in the presence of inhibitors, high-molecular-mass fragments remain uncleaved). The presence of potential inhibitors is usually assessed by using photometry at 260 and 280 nm. In a good DNA preparation, the A_{260}/A_{280} ratio should be in the range of 1.75–2.0; otherwise, the contamination (e.g., with residual protein or phenol) may be too high. Photometric measurement alone does not allow conclusions to be made regarding the integrity of the DNA.

The fastest method for controlling the quality of the total RNA preparation is agarose gel electrophoresis under nondenaturing conditions, as was applied in the separation of DNA. In case of doubt, however, the RNA should be run on an agarose gel under denaturing conditions to check its integrity. Ideally, the three major ribosomal RNA species (28S, 18S, and 5S) will be detectable as relatively sharp bands. Bands smeared to lower molecular masses or absence of the bands strongly indicates decay of RNA. A densitometric measurement of ribosomal RNA bands with indexing may become an in-laboratory standard for assessing the quality of RNA preparations; assessment of peak asymmetry with peaks trailing to lower molecular masses also is a suitable indicator of RNA integrity. In addition, agarose gel electrophoresis will indicate the degree of DNA contamination in the RNA preparation. For these reasons, the photometric measurement alone does not allow one to draw conclusions as to the integrity of the RNA.

4.1.2. Controls for cDNA synthesis and amplification.

Preparations for PCR amplification must be controlled carefully with reaction-internal as well as reaction-external positive control specimens to check for enzymatic activity and to exclude the presence of putative inhibitors. Negative controls are essential for monitoring contaminations. In general, the following control reactions should be distinguished: The positive-control reaction supplies information about enzymatic activity and inhibitors in the sample; the negative-control reaction detects contamination with human DNA (e.g., from the investigator) or with PCR product; and the reagents-control reaction indicates whether any of the reagents are contaminated.

4.1.2.1. cDNA synthesis. The crucial control for monitoring the performance of cDNA synthesis is the use of an internal reaction control. cDNA synthesis can proceed either from mRNA transcripts, which are present in each RNA preparation (i.e., mRNA of ubiquitously expressed, so-called housekeeping genes such as transcripts for ribosomal proteins), or from RNA, which is added to the sample as an internal standard at the time of preparation. Internal controls for synthesis are positive controls that lead to a defined product; if the amplification is not successful, the controls indicate a degradation of the RNA, faulty priming during the cDNA synthesis, or absence of enzyme activity.

The addition of a defined number of molecules of an amplification control permits determination of the

lesser sensitivity of the RT-PCR. This is particularly important in PCR applications of high sensitivity. A mandatory contamination control for RT-PCR assays consists of a reaction with the RT omitted. In summary, equivalent amplification characteristics of control and template must be assured, and the control must be clearly distinguishable from the template.

4.1.2.2. PCR reaction. *Internal reaction controls* are positive controls and are particularly important in cases where the presence or absence of an amplification product is diagnostically relevant, i.e., with gene deletions or Y-chromosomal sequences. The negative result must be clearly distinguishable from a technical failure of the assay. Thus, to guarantee that amplification is successful under a given set of reagents and DNAs, one can use a control target gene essential for the organism to survive. Such genes are present in at least the hemizygous state, because their absence from the genome is lethal (i.e., so-called lethality factor). A good example is the gene for the vitamin D-binding plasma protein (20); its polymorphisms have been extensively examined in many ethnic groups worldwide, and no homozygous loss of gene activity has ever been identified. Therefore, a coamplification of a segment of this gene by the PCR will be in all cases successful and will demonstrate the successful amplification reaction.

External positive controls of appropriate DNA and appropriate dilutions thereof allow the quality of the reaction solution to be checked, and information regarding the detection limit and specificity of the PCR are obtained. The same master mix solutions used for the diagnostic test (i.e., patient's material) must be used in the external positive controls. Amplification controls are to be performed with each reaction if the detection limit of the procedure is suspected to be inadequate to detect the product. Controls of this type increase the contamination risk in the test series, if the nucleic acid used corresponds to a positive control sample. Vector-cloned target sequences or a genomic DNA of known copy number is suitable.

An *external negative control (contamination control)* must be performed in each PCR test; indeed, several controls should be contained in one run, one for each primer pair applied. In general, these controls are to furnish information about the point in time at which a contamination occurs in the course of a PCR-based test. A blank reaction vessel taken through the entire course of the sample preparation comes into contact with all solutions used in the preparation, but contains no amplifiable material (so-called mock preparation). If necessary, different mock preparations can be integrated at various preparation stages in the course of the nucleic acid preparation, if necessary. In this manner the individual steps at which contamination can occur can be identified. Mock controls allow assessment of the overall quality of the PCR test.

In addition, during the pipetting process, when bringing the samples into contact with the enzymatic amplification solution, water samples have to be added: These contain all reaction components, but water is used instead of the sample. Water samples must be present at least at the start and at the end of a pipetting series in which sample material is processed. The widely practiced method of detecting the absence of contamination solely by means of the water sample is not admissible, because this does not detect contamination sources in the course of sample handling; water can serve as a control only of the amplification reagents.

If the same amplification assay is to be repeated—as will be the case in most diagnostic tests—all

contamination controls must be checked regularly by means of specific hybridization with use of Southern transfer or dot-blot. In PCR tests that amplify RNA targets, a contamination control should be performed with the RT step omitted. In this way contamination caused by DNA fragments from previous amplifications can be detected.

4.1.3. Controls for the evaluation of test results.

4.1.3.1. Control of restriction digestion. Digestion of genomic DNA can be reduced by inhibitors of the activity of enzymes present in the preparation, by inappropriate reaction conditions, or by low enzymatic activity. The digestibility of genomic DNA can be assessed through agarose gel electrophoresis. The following criteria serve for an assessment of a successful restriction:

- 1) After restriction digestion with most enzymes, a continuous, mostly smeared band is observed from the high- to low-molecular-mass range. This reflects the size heterogeneity of the restricted genomic fragments. Where digestion is not successful, a pronounced high-molecular-mass fraction will persist at the molecular range corresponding to the uncut DNA. With some enzymes, a high-molecular-mass fraction may be observed even after extended digestion incubation times. Thus, although disappearance of the high-molecular-mass fraction indicates full enzymatic activity, persistence of this fraction does not necessarily indicate insufficient digestion.
- 2) An elegant method to assess inhibitors in the sample is to branch off an aliquot of the digestion mix, and then add to this a known amount of high-molecular-mass molecular marker (see *Section 4.1.3.2*). For example, together with the genomic nucleic acids, the 48-kb large λ -bacteriophage (e.g., 1 μ g) is digested into its predicted fragments. One can monitor the suitability of the sample DNA on the basis of the digestibility of the indicator DNA in the aliquot by agarose electrophoresis after different time intervals and extend the digestion times of the genomic DNA if necessary (9)(21).
- 3) The human genome contains repetitive sequences, e.g., mitochondrial DNA, which may appear as distinct sharp bands (satellite bands) within the background smear of heterogeneous fragments. Satellites, therefore, do indicate successful restriction; however, not all enzymes will generate a satellite band pattern.

Restriction digestion of PCR products is often applied to identify mutations on the basis of various restriction fragment lengths. Compromised enzymatic activity in these cases leads to false interpretation of results. Complete digestion of PCR products can be controlled only by a second invariant restriction site of the same enzyme in the PCR product to be tested. This must be strictly observed in the construction of the diagnostic fragment, to assure a correct interpretation of results.

4.1.3.2. Control of electrophoresis. Quality assessment of electrophoretic methods involves, in the first place, calibrators for length and suitable control fragments at defined concentrations; controls must be subject to the same sample preparation procedures as the diagnostic specimens. Length calibrators permit determination of the size of PCR products, and defined concentrations help control the detection limit of the visualization process used. Many manufacturers offer good calibrators for length in different molecular mass ranges, so-called basepair ladders; these often permit an exact size determination of the

electrophoretically separated fragments.

Previously restricted vector DNAs also can be used as molecular mass markers, given that the digestion of vector genomes (plasmids or phages) results in defined band patterns. Because the DNA fragments are generated from the same molecule, all the fragments exist in equimolar concentrations. From the known DNA quantity added into the digestion, one can calculate the DNA quantity for each fragment as a fraction of the uncut DNA amount. Because the ethidium bromide fluorescence is proportional to the DNA quantity, and because the relative quantity of the individual bands of the calibrator is known, the concentration of PCR fragments can be judged in comparison with the fluorescence intensity of the bands of the calibrators. Many manufacturers or distributors of restriction enzymes list the precise restriction fragment lengths of vector genomes in the annexes to their manuals. Finally, basepair ladders and restricted vector calibrators can be mixed to increase the resolution of the calibration curve.

4.1.3.3. Control of blotting and of hybridization. In blot hybridizations, positive and negative controls should always be analyzed on the same membrane alongside the patient's sample reactions. This excludes misinterpretation from the use of different hybridization conditions in different reaction vessels.

4.1.3.4. Control of sequencing. Manufacturers usually supply suitable DNA amplification templates, and matching primers, as controls for their sequencing reagents. Moreover, this sequencing reaction can help control the quality of the sequencing gel electrophoresis. For analysis of mutations, the wild-type allele and samples from family members should be sequenced in parallel.

4.2. external quality control (proficiency testing)

Considering the multitude of methodological variants and diagnostic approaches, it does not appear feasible to set up external quality-assessment trials for every diagnostic problem, especially if the diseases considered are rare. This problem is particularly pertinent in smaller countries. For this reason, the Central Reference Institution of the German Society of Clinical Chemistry has started to perform its first external quality-assessment trials with two major objectives: methodological proficiency testing and application-based proficiency testing.


Methodological proficiency testing is intended to control the quality of the elementary analytical steps in molecular genetic diagnosis: the DNA/RNA preparation, the performance of the PCR method with supplied "standard primers," and the agarose gel electrophoresis. Application-based proficiency testing is at this time suitable for relatively frequent diagnostic questions, e.g., the factor V Leiden mutation in thrombophilia, caused by the resistance of clotting factor V to activated protein C; hereditary hemochromatosis; α_1 -antitrypsin deficiency; mutations in apolipoproteins B100, E2, E3, and E4; and mutations in LDL receptor, β -myosin heavy chain, and others. These will increasingly be included in nationwide quality-assessment trials in Germany. Obviously, the above-mentioned favorably complement the conventional routine panel of corresponding analytes/markers commonly determined in clinical chemistry laboratories. Continuous cell lines obtained from diseased individuals or stably transfected with genes coding for the respective gene products could be used as standardized template

sources for proficiency testing in molecular diagnostics laboratories. For rarer genetic diseases, international trials make more effective use of resources and expertise.

In conclusion, during recent years laboratory science has faced an increasing interest in molecular diagnostics and a corresponding demand for routine genetic testing. Expectations are high for two reasons: First, much is expected from molecular testing—expectations nurtured by scientific progress in, e.g., the Human Genome Project. Second, physicians have become used to a high quality of test results from the routine clinical laboratory through their day-to-day use of more-conventional laboratory markers. Although molecular tests to support clinical diagnostics will arise and prove useful over time, the issues important for the laboratories are already defined. A rapidly increasing number of laboratories are now establishing molecular technologies to use for clinical diagnosis. This results in an obvious need for standardization of both the test systems and the laboratory procedures, and efforts should be made towards this end. Particularly for the amplification-based techniques, internal laboratory procedures have to be carefully controlled. Because both the technology and its applications are in constant change and development, we emphasize that this publication is intended to recommend, not define, good laboratory practice and internal quality control at this time and to guide troubleshooting, primarily in diagnostic amplification techniques. Communication of operating procedures and scientific discussion of them is important for such guidelines to evolve. Only then will these procedures become general tools suitable for maintaining and strengthening confidence in molecular clinical testing.

► Footnotes

IFCC document, stage 1, draft 1.

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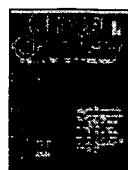
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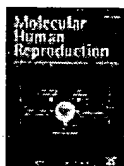
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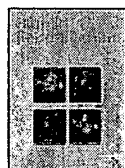
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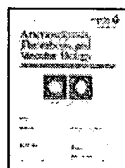
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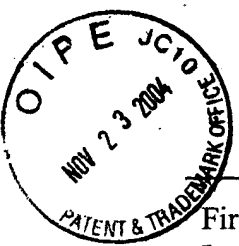
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Appln. No. : 09/932,888

Filed : August 20, 2001

Title : Porcine Leptin Protein, Antisense and Antibody

Docket No. : LL31.12-0016

Group Art Unit: 1647

Examiner: C. J. Saoud

EXHIBIT D

of

DECLARATION

submitted under 37 C.F.R. 1.132

Hybridization with Radioactive Probes: Using DNA Fragments as Probes.
Current Protocols in Molecular Biology. Section II 6.3.5. (2000)

HYBRIDIZATION WITH RADIOACTIVE PROBES

After plaques or colonies have been transferred to nitrocellulose filters, the desired clone can be detected by its ability to hybridize to a DNA probe. This is a rapid, effective screening procedure that allows the identification of a single clone within a population of millions of other clones. The filters are hybridized with a ^{32}P -labeled nucleic acid probe, the excess and incorrectly matched probe is washed off the filter, and the filter is autoradiographed. Two features of the nucleic acid probe used for these experiments are critical to the successful screening of recombinant DNA libraries. First, the probe must hybridize only to the desired clones and not to any other clones. Thus, the nucleic acid sequence used for a probe must not contain any reiterated sequences or sequences that will hybridize to the vector. Second, the specific activity of the probe must be at least 10^7 cpm/ μg . Most of the procedures for labeling DNA or copy RNA molecules are described in Chapter 3, and a support protocol is presented here that allows the 5' end-labeling of a mixture of oligonucleotides.

The two basic protocols presented in this section describe steps required to hybridize labeled probes to recombinant DNA clones on filters. Two protocols are presented because conditions for hybridizing short oligonucleotide probes and longer nucleic acid probes to filters are different.

UNIT 6.3

BASIC PROTOCOL

Using DNA Fragments as Probes

HYBRIDIZATION IN FORMAMIDE

Bacteriophage plaques or bacterial colonies bound to a filter membrane are detected by hybridization with a radioactive probe. Hybridization proceeds on prewet filters placed in a sealable plastic bag. After hybridization the filters are removed from the sealed bag, excess probe is washed off, and the filters are autoradiographed to identify the clones that have hybridized with the probe.

Materials

Nitrocellulose membrane filters bearing plaques, colonies, or DNA (UNITS 6.1 & 6.2)
 Hybridization solution I
 Radiolabeled probe, 1 to 15 ng/ml (UNIT 3.5)
 2 mg/ml sonicated herring sperm DNA
 High-stringency wash buffer I
 Low-stringency wash buffer I
 Sealable bags
 42°C incubator
 Water bath adjusted to washing temperature (see commentary)
 Glass baking dish
 Additional reagents and equipment for autoradiography (APPENDIX 3)

Incubate filters with probe

1. Wet filters with hybridization solution I. Lay a filter membrane bearing plaques on top of 5 to 20 ml of hybridization solution I and allow solution to seep through filter. It is important to wet only one surface at a time to prevent trapping air in filter. Wet each filter in turn, producing a stack of wet filters.

When multiple filters are to be hybridized to the same probe, no more than twenty 8.2-cm discs or ten 20 × 20 cm square filters should be placed in one stack.

Using DNA
Fragments
as Probes

6.3.1

Estimate the volume of hybridization solution used to wet the filters; this is a significant fraction of the volume of the hybridization reaction.

2. Transfer the stack of wetted filters to an appropriately sized sealable bag. Add enough hybridization solution to generously cover filters and seal.

Note the volume of hybridization solution used to cover the filters.

3. Prehybridize filters by placing the bag in a 42°C incubator for at least 1 hr.

Some investigators omit this step.

4. While filters are prehybridizing, pipet the radioactive probe into a screw-cap tube, add 2 mg (1 ml) sonicated herring sperm DNA, and boil 10 min. Place boiled probes directly into ice to cool.

The amount of probe used is important, and should be in the range of 1 to 15 ng/ml of hybridization reaction. The volume of the hybridization reaction can be assumed to be the amount of hybridization solution added to the filters.

5. Add 2 ml hybridization solution I to the boiled probe.

6. Remove bag containing filters from the 42°C incubator. Open bag, add probe mixture, exclude as many bubbles as possible, and reseal.

A good way to add the radioactive probe is to take it up in a syringe with an 18-G needle and then inject it into the bag. Reseal the bag after adding probe.

7. Mix probe in the bag so that filter is evenly covered. Replace bag in the 42°C incubator and let hybridize overnight.

Wash filters to remove nonhybridized probe

8. Warm 1 liter high-stringency wash buffer I to the "washing temperature" in a water bath.

The stability of washing temperature and salt concentrations are critical features of this experiment. See discussion in commentary.

9. Remove bag containing hybridizing filters from the 42°C incubator. Cut bag open and squeeze hybridization solution out of the bag.

CAUTION: *Handle material carefully as it is extremely radioactive. This should be done on disposable paper bench covers.*

10. Quickly immerse the filters in 500 ml low-stringency wash buffer I at room temperature in a glass baking dish. Separate all the filters, as they may stick together during hybridization.

The volume of the low-stringency wash buffer is not important as long as the filters are completely covered. The filters must not be allowed to dry as the radioactive probe will irreversibly bind the filters if the filters dry in contact with probe. (The type of container used to hold the filters is not important as long as it transfers heat well. Thus glass, metal, or enamel containers are better than plastic.)

The low-stringency wash only removes nonhybridized probe formamide and hybridization solution; it does not determine the stringency of the hybridization.

11. Rinse the filters three times with 500 ml low-stringency wash buffer. Let the filters sit 10 to 15 min at room temperature in low-stringency wash buffer with each rinse.
12. Pour off the low-stringency wash buffer and pour in 500 ml high-stringency wash buffer (prewarmed to washing temperature).

13. Replace the high-stringency wash buffer with another 500 ml of high-stringency wash buffer, then place the glass dish containing the filters in incubator at wash temperature. Make sure that the temperature in the glass dish reaches the desired washing temperature by placing a thermometer directly into the bath and measuring the temperature. Usually 15 to 20 min at the desired wash temperature is sufficient to remove most of the background radioactivity.

Of course, if the glass dish is placed in a water bath, be careful that the water from the water bath does not get into the filters.

Autoradiographing filters

14. Remove filters and mount them either wet or dry on a plastic backing. If the filter(s) is to be exposed wet, then isolate it from the film by covering it with plastic wrap.

Used X-ray film provides a good form of plastic backing for filters.

15. Mark the filters with radioactive ink to assist in alignment and autoradiograph.

An easy way to apply radioactive ink is to mark adhesive-backed paper labels with radioactive ink and then attach the stickers to the plastic wrap cover.

X-ray intensifying screens greatly decrease the amount of exposure time required.

ALTERNATE PROTOCOL

HYBRIDIZATION IN AQUEOUS SOLUTION

This method differs mainly in that formamide is not used in the hybridization solution. Follow the basic protocol except use the reagents and alternate parameters listed below.

Additional Materials

Hybridization solution II
Low-stringency wash buffer II
High-stringency wash buffer II
65°C incubator

1. Prehybridize as in basic protocol except that the filters are prehybridized at 65°C using hybridization solution II.

Hybridization solution II may have to be prewarmed to solubilize the SDS.

2. Prepare probe as in step 4 of basic protocol and dilute with 2 ml of hybridization solution II.
3. Hybridize overnight as in steps 6 and 7 of basic protocol except use a hybridization temperature of 65°C.
4. Remove bag containing hybridization from the 65°C incubator. Squeeze out the hybridization solution, taking care to avoid contamination with the excess radioactive hybridization solution.
5. Immediately rinse filters twice with low-stringency wash buffer II.

It is unnecessary to maintain a given temperature for this wash; just let the filters sit in wash buffer at room temperature until ready to proceed.

6. At 65°C, proceed to wash filters with high-stringency wash buffer II. Employ multiple quick washes (5 to 8) and immerse filter in a final wash for ~20 min. Check the radioactivity of the filters with a Geiger counter and be certain that they produce a signal only a fewfold above background levels.

REAGENTS AND SOLUTIONS

High-stringency wash buffer I

0.2× SSC (APPENDIX 2)
0.1% sodium dodecyl sulfate (SDS)

High-stringency wash buffer II

1 mM Na₂EDTA
40 mM NaHPO₄, pH 7.2
1% SDS

Hybridization solution I

Mix following ingredients for range of volumes indicated (in milliliters):

Formamide	24	48	72	120	240	480
20× SSC	12	24	36	60	120	240
2 M Tris-Cl, pH 7.6	0.5	1.0	1.5	2.5	5.0	10
100× Denhardt's solution	0.5	1.0	1.5	2.5	5.0	10
Deionized H ₂ O	2.5	5.0	7.5	12.5	25	50
50% dextran sulfate	10	20	30	50	100	200
10% SDS ^a	0.5	1	1.5	2.5	5	10
Total volume	50	100	150	250	500	1000

^aIn place of SDS, *N*-lauroylsarcosine (Sarkosyl) may be used.

Add the SDS last. The solution may be stored for prolonged periods at room temperature.

The dextran sulfate should be of high quality. Pharmacia produces acceptable-grade dextran sulfate. Recipes for SSC and Denhardt's solution are in APPENDIX 2.

Hybridization solution II

1% crystalline BSA (fraction V)
1 mM EDTA
0.5 M NaHPO₄, pH 7.2 (134 g Na₂HPO₄·7H₂O plus 4 ml 85% H₃PO₄/liter = 1 M NaHPO₄)
7% SDS

Low-stringency wash buffer I

2× SSC (APPENDIX 2)
0.1% SDS

Low-stringency wash buffer II

0.5% BSA (fraction V)
1 mM Na₂EDTA
40 mM NaHPO₄, pH 7.2
5% SDS

Sonicated herring sperm DNA, 2 mg/ml

Resuspend 1 g herring sperm DNA (Boehringer Mannheim #223636) in a convenient volume (about 50 ml of water) by sonicating briefly. The DNA is now ready to be sheared into short molecules by sonication. Place the tube containing the herring sperm DNA solution in an ice bath (the tube must be stable even if the ice begins to melt). The sonicator probe is placed in the DNA solution (without touching the bottom of the vessel). The sonicator is turned on to 50% power 20 min, or until there is a uniform and obvious decrease in viscosity. At no time should the tube containing the DNA become hot to the touch. After sonication, the DNA is diluted to a final concentration of 2 mg/ml, frozen in 50-ml aliquots, and thawed as needed.

COMMENTARY

Background Information

All hybridization methods depend upon the ability of denatured DNA to reanneal when complementary strands are present in an environment near but below their T_m (melting temperature). In a hybridization reaction involving double-stranded DNA on a filter and a single-stranded DNA probe there are three different annealing reactions occurring. First, there are the desired probe-DNA interactions which result in signal. Second, there are mismatch interactions that occur between related but non-homologous sequences; these mismatch hybrids are the ones that must be eliminated during the washing of the filters. Non-sequence-specific interactions also occur and these result in noise. The ability to extract information from a particular hybridization experiment is a function of the signal-to-noise ratio. High background or poor specific signal can both result in uninterpretable results.

Washing nitrocellulose filters is required to remove excess radioactive probe, as well as radioactive probe that has bound to the DNA on the filter as mismatch hybrids. Temperature and salt concentration dramatically affect the maintenance of specific hybrids. Detergents and other charged species can have a profound effect upon the nonspecific binding of species that contribute to background. In this protocol, hybridization is achieved in a solution containing 50% formamide. Excess probe is rinsed away under low-stringency conditions so that further hybridization will not occur. Once the hybridization solution is rinsed away, it is possible to proceed to a high-stringency wash without fear of further hybridization. When washing is complete, the filters should produce very little "noise" when monitored with a Geiger counter. Although single-copy sequence probe normally does not produce a signal that is detectable with a Geiger counter, a probe corresponding to more abundant sequences will produce a signal that can be "heard" with a Geiger counter.

Literature Review

Hybridization to filter membranes forms a basis of recombinant DNA technology and is described in detail earlier in the manual (UNIT 2.9). The protocols described here vary from those used for Southern blot filter hybridization in that the volume of the hybridization is usually larger and the washing conditions are different. Dextran sulfate is an important component of the hybridization

solution as it increases the rate of reassociation of the nucleic acids.

The protocols in this unit describe methods for hybridizing radioactive probes to membrane-bound plaques or colonies. These procedures for screening recombinant clones were first suggested by Grunstein and Hognes (1975) and by Benton and Davis (1977). The conditions of hybridization proposed in the basic protocol involving hybridization in formamide was originally described by Denhardt (1966) and Gillespie and Spiegelman (1965) while the alternate protocol using aqueous hybridization solution was introduced by Church and Gilbert (1984).

The method of washing filters under stringent conditions to remove background was first proposed by Southern (1975). Botchan et al. (1976) described the benefit of adding SDS to the wash solution. Jeffreys and Flavell (1977) first employed the wash conditions described in the protocols presented here.

Critical Parameters

Hybridization. Kinetically, the hybridization of DNA (or RNA) probes to filter-bound DNA is not significantly different from hybridization in solution. For single-stranded probes, the rate of hybridization follows first-order kinetics, since probe is available in excess. Under conditions of excess probe, the time for hybridization is inversely proportional to the probe concentration. For double-stranded probes the rate of hybridization displays a more complex relationship to the initial probe concentration. However, to a first approximation the initial probe concentration is inversely proportional to the rate of hybridization. To determine the actual time required for the successful hybridization of a given probe, either empirical data must be available or the following formula can be used to determine the length of time (in hours) required to achieve 50% hybridization (T_{50}):

$$1/x \times 1/y \times 1/z \times 2 = T_{50}$$

where x is the weight of probe in micrograms; y is the complexity of probe in kilobases; and z is the volume of hybridization solution in milliliters. The length of time T is given in hours. Maximum hybridization signal will be obtained if the reaction is allowed to proceed to $5 \times T_{50}$, although 1 to $2 \times T_{50}$ is often used.

It is also clear that nonspecific interactions

occur and that in any hybridization, sources of noise will be present. Therefore, from a practical standpoint one conventionally utilizes concentrations of nick-translated probe on the order of 1 to 15 ng/ml of hybridization, where the specific activity of the probe is from 5×10^7 cpm/ μ g to $>10^8$ cpm/ μ g. Too much probe in a hybridization is as bad as too little.

One important source of background hybridization to filters is due to the hybridization of the probe to vector sequences or to *E. coli* DNA. Be certain that there is no vector or *E. coli* DNA sequences in the probe. This can best be ensured by isolating the probe from one type of vector (e.g., plasmid) and screening a library made with a different type of vector (e.g., bacteriophage).

Washing temperature. Washing at low stringency is a straightforward proposition. Buffer is added at room temperature and washing proceeds at room temperature.

High-stringency wash is determined empirically. The relative homology between the probe and target sequence is a determining parameter. If the homology is 100%, a high temperature (65° to 75°C) can be used. As the homology drops, lower washing temperatures must be used. In general one starts at 37° to 40°C, raising the temperature by 3° to 5°C intervals until background is low enough not to be a major factor in the autoradiography.

The length of the probe is also important. Very short probes (<100 bp) must be washed at lower temperatures, even if the homology is 100%. Washing strategy is the same as for probes of differing homology.

Salt concentration. The lower the salt concentration, the higher the stringency. With this said, the protocols as outlined do not require adjustment of salt concentration for adjustment of stringency. Only the washing temperature is varied.

Probe. The nucleic acid probe must be of high specific activity and greater than 50 bp in length so that it can form stable hybrids.

Anticipated Results

After washing the filters the background

should be barely detectable with a Geiger counter.

With a high-specific-activity probe $>5 \times 10^7$ cpm/ μ g and an overnight hybridization reaction with a 1-kb unique sequence probe, hybridizing bacterial colonies or bacteriophage plaques can be visualized after a 1 to 18 hr exposure.

Time Considerations

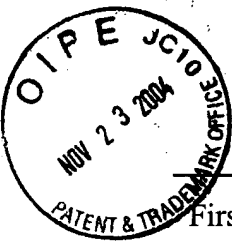
Generally hybridizations are carried on overnight for 12 to 16 hr. This is sufficient for most probes and blots. However, with probes of increasing complexity longer hybridization times are required. This is preferable to increasing the probe concentration from experiment to experiment.

Autoradiography requires 1 to 18 hr.

Literature Cited

- Benton, W.D. and Davis, R.W. 1977. Screening λ gt recombinant clones by hybridization to single plaques in situ. *Science* 196:180.
- Botchan, M., Topp, W., and Sambrook, J. 1976. The arrangement of simian virus 40 sequences in the DNA of transformed cells. *Cell* 9:269-287.
- Church, G. and Gilbert, W. 1984. Genomic sequencing. *Proc. Natl. Acad. Sci. U.S.A.* 81:1991-1995.
- Denhardt, D. 1966. A membrane filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* 23:641-646.
- Gillespie, D. and Spiegelman, S. 1965. A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. *J. Mol. Biol.* 12:829-842.
- Grunstein, M. and Hogness, D. 1975. Colony Hybridization: A method for the isolating of cloned DNA's that contain a specific gene. *Proc. Natl. Acad. Sci. U.S.A.* 72:3961.
- Jeffreys, A.J. and Flavell, R.J. 1977. A physical map of the DNA region flanking the rabbit β globin gene. *Cell* 12:429-439.
- Southern, E.M. 1975. Detection of specific sequence among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503-517.

Contributed by William M. Strauss
Harvard Medical School
Boston, Massachusetts



First Named

Inventor : Michael E. Spurlock

Appln. No. : 09/932,888

Filed : August 20, 2001

Title : Porcine Leptin Protein, Antisense and Antibody

Docket No. : LL31.12-0016

Group Art Unit: 1647

Examiner: C. J. Saoud

EXHIBIT E

of

DECLARATION

submitted under 37 C.F.R. 1.132

Course Instructions posted at <http://catlserver.tamu.edu/ning/ing626.htm> for Lab Session No. 6,
Fall 2003: Course Entitled Gene Expression (ANSC/GENE 626 (edited by. N. Ing 9/2/03));
Nancy H. Ing, Instructor; Texas A & M University; College Station, Texas

Fall 2003: GENE EXPRESSION

ANSC/GENE 626 (edited by. N. Ing 9/2/03)

Nancy H. Ing, Instructor

Kleberg 410D, 862-2790

ning@cvm.tamu.edu

The purpose of this course is to provide graduate students with experience in working with RNA and DNA and with the theories behind the use of molecular biology in research.

Class will be held in BICH 243 in Fall semester on Thursdays (lecture from 12:40 to 1:35 p.m.) and Fridays (lab from 12:45 to 3:40 p.m.)

9/04	Lecture 1:	Introduction to the course, Safety rules What is gene expression? What is a gene? What does it do?
9/05	Lab 1	Introduction: Pipetting, Restriction digestion of plasmid DNA.
9/11	Lecture 2	What are plasmids and how are they used? Electrophoretic analysis of macromolecules.
9/12	Lab 2.	Plasmid DNA analysis by agarose gel electrophoresis
9/18	Lecture 3	Engineering and amplifying DNA in basic and specialized vectors
9/19	Lab 3.	Ligation of gel-purified DNA fragments
9/25	Lecture 4:	Growing and preparing plasmid DNA. Other DNA preps.
9/26	Lab 4	Transformation of E. coli
10/2	Lecture 5:	Restriction enzymes and analysis of plasmid DNA
10/3	Lab 5.	Plasmid minipreps
10/9	Lecture 6:	How can you identify a DNA specifically? (two ways)
10/10	Lab 6:	Restriction analysis of plasmid DNA minipreps
10/16	Lecture 7:	What does DNA sequence tell you? Functions of gene sequences
10/17	Lab 7	DNA sequencing with PCR
10/23	Lecture 8	Transcription in cells and out
10/24	Lab 8:	Analyzing DNA sequences
10/30	Lecture 9	What natural types of RNA are in cells? How do you analyze RNA? What are their functions?
10/31	Lab 9:	Making a DNA template for in vitro transcription of an sense RNA
11/6	Lecture 10:	Acrylamide gel electrophoresis for small RNA and protein analyses
11/7	Lab 10	In vitro transcription and PAGE analyses
11/13	Lecture 11	Translation in cells and in vitro

11/14	Lab 11:	In vitro translation and making SDS-PAGE gels
11/20	Lecture 12	Analyses of proteins by SDS-PAGE
11/21	Lab 12	Running protein samples on SDS-PAGE and staining
11/27 and 28		HAPPY THANKSGIVING!!!
12/ 4	Lecture 13	Other Analysis of proteins
12/5	Lab 13:	Destaining and Analyzing the SDS-PAGE results.
11/21		11A. Analysis of Extracted RNA by A260; In vitro transcription of cRNA probes Overnight cultures

13. Plasmid DNA restriction and gel analysis

Protocols will be provided. Required viewing of videos. Required reading from Gerstein "Molecular Biology Problem Solver" as well as other materials relating to kits, etc used. Students will be evaluated on preparation and participation (33%), lab notebook (33%) and a written laboratory report (33%) due the Friday after the last class.

ANSC/GENE 626 REQUIRED READING & VIDEOS

- Required Text: Gerstein "Molecular Biology Problem Solver" 2001
- Lab 1 Pipettors pp 67-77.
- Lab 2 RNA purification pp 197-224
- TriPure handout - see Roche website at <http://biochem.boehringer-mannheim.com>
- Isolation of mRNA Video*
- Lab 3 SPectrophotometers pp 94-111
- Ambion Maxiscript and Northernmax (formaldehyde gel) Protocols - see ambion website at www.ambion.com
- Lab 4 Gel Electrophoresis pp331-371
- Polyacrylamide Gel Electrophoresis Video
- Lab 5 Nucleic acid hybridization pp 399-460
- Lab 6 DIG RNA labeling kit instructions from Roche (see website biochem.roche.com)
- Southern Blot Hybridization Video
- Lab 7 DIG luminescent detection kit from Roche
- Lab 8 Clontech MIMIC kit information
- Polymerase Chain Reaction pp291-330
- PCR and Detection Video
- Lab 9 Agarose Gel Electrophoresis Video
- Lab 10 DNA Ligation Video
- Lab 11 Transformation Video
- Lab 12 DNA Purification pp167-195
- Rapid Isolation of Plasmid DNA (minipreps) Video
- Lab 13 Restriction enzymes pp225-266

*Videos may be checked out from Kleberg 410A for 1 h during the day or overnight after 4PM (returned by 9AM). Single and group viewing is available in the Multi Media Center (Kleberg 023).

LAB RULES

A. GENERAL

1. Everyone is individually responsible for the experiments. Come prepared by reading protocols and required reading in advance!! Activities will be started immediately, while explanations and discussion sessions will occur as time permits. COME PREPARED to ASK QUESTIONS, especially during discussions.
2. Equipment in this and neighboring labs is shared. Know or ask how to use it. Obey user rules, such as signing logs. Leave all equipment in good working order. If there are problems, tell someone so we can fix them!
3. Leave the lab better than you found it. Wash your own glassware, clean up your work area, write the names of reagents that are running out on the "to be ordered" list, etc.
4. Lab notebooks are bound volumes, are kept in pen with numbered, dated pages. They are designated only to that purpose, are labelled on the cover with Name, dates and lab, and are only removed from the lab with instructor permission. In them, each person describes their activities and observations each day. Each page should be dated. Each photo or X-ray film should be labeled with initials, date, and identification of gel type, lane contents and dye migration. Record things chronologically along with dates. Start a new project on a new page with a description of its purpose. A stranger should be able to pick up your notebook and understand how and why you did an experiment. Protocols don't have to be written each time, but may be referred to, instead. For this, you may consider the handouts you receive in lab as references. Use them as such and refer to specific pages (dates) within them. Record your activities and observations clearly, using complete and understandable sentences. Write down your reagents, including buffers and buffer recipes. KEEP UP WITH NOTEBOOK ENTRIES EVERY DAY...otherwise, data will be lost!
5. All reagents and samples saved must be labeled with the date; your initials, and WHAT IT IS. In my lab, we use simple sample numbers, such as 1 - 10. To key it into our notebook, we write: lab notebook #, page #; (NI2P30 relates to Nancy Ing Book 2, page 30), so the notebook, not the tube, is where the full description of the sample exists. Items not labeled sufficiently may be discarded.
6. Store things in appropriate places! For plasmids and reactions and buffers, store at -20C in storage box provided to your group unless otherwise noted. Note storage places in your notebook.

B. SAFETY IS THE #1 PRIORITY

1. The only safety activity not strictly enforced is wearing safety glasses: this is a good idea but is not mandatory. Wearing a lab coat is mandatory and wearing gloves will become a habit (see below).
2. Working with open flames and hazardous chemicals have strict safety protocols - ask for them and follow them.

3. Working with radioactivity is a privilege, not a right. Workers must monitor for contamination, before, during, and after the procedure. Radiation safety training is required. WE RUN A CLEAN LAB.

4. Because we work with HAZARDOUS SUBSTANCES, there is NO EATING, DRINKING, SMOKING, or APPLYING MAKE-UP in the lab.

5. Garbage must be disposed of properly. Glass and sharps, biohazard, chemical, and radioactive waste must be separated from the rest.

C. GOOD LAB TECHNIQUES

1. ICE IS NICE! Work on it unless otherwise directed. It slows degradation of macromolecules.

2. Many reagents settle on storage, so mix them! All frozen solutions need to be thawed and mixed before using.

3. ENZYMES DO OUR WORK. They are stable as glycerol solutions at -20°C. Keep them in the freezer as much as possible. Only remove them in -20°C blocks. DO NOT WARM ENZYME STOCKS! When pipetting small amounts of viscous solutions like enzymes, check loaded pipet tip and evacuated one to assure that enzyme got into the reaction. After addition, mix reaction solution gently but thoroughly: can pipet total volume up and down OR vortex gently and flash spin in microfuge to return reaction to the tube bottom.

ADA Statement, Copyrights, and Plagiarism

The Americans with Disabilities Act (ADA) is a federal antidiscrimination statute that provides comprehensive civil rights protection for persons with disabilities. Among other things this legislation requires that all students with disabilities be guaranteed a learning environment that provides for reasonable accommodation of their disabilities. If you believe you have a disability requiring an accommodation, please contact the Dept. of Student Life, Services for Students with Disabilities in Room 126 of the Koldus Bldg. or call 845-1637.

Copyrights

The handouts used in this course are copyrighted. By handouts", I mean all materials generated for this class, which include but are not limited to syllabi, quizzes, exams, lab problems, in-class materials, review sheets, and additional problem sets. Because these materials are copyrighted, you do not have the right to copy the handouts unless I expressly grant permission.

Plagiarism

As commonly defined, plagiarism consists of passing off as one's own ideas, words, writings, etc., which belong to another. In accordance with this definition, you are committing plagiarism if you copy the work of another person and turn it in as you own, even if you should have the permission of that person. Plagiarism is one of the worst academic sins, for the plagiarist destroys the trust among colleagues, without which research cannot safely be communicated.

If you have any questions regarding plagiarism, please consult the latest issue of the Texas A&M University Student Rules, under the section "Scholastic Dishonesty."

BICH/GENE 432

Safety Precautions

1. **ABSOLUTELY** no food or drink in the lab. This includes gum.
2. Always wear gloves when working with Ethidium bromide, radioactive compounds, acrylamide and organic compounds.
3. Always wear UV safety glasses when using UV illumination.
4. Use special care when working with open flames. Don't forget to turn off gas after use.
5. Clean up spills immediately. Notify instructor if hazardous compounds are spilled.
6. Discard organic solutions in appropriate bottles.
7. Discard Ethidium bromide waste in correct container.
8. All culture medium and labware used for bacteria needs to be autoclaved or put in Chlorox before disposal or washing.
9. After using radioactive materials, wash the work area and clean spills immediately. Dispose of gloves in radioactive waste as soon as you are finished.
10. All sharps (including broken glass, needles and razor blades) should be disposed in clearly marked containers, not in the general trash.
11. If you have questions about anything, **ASK!**
12. Lab coats are required!

LAB REPORTS - BICH/GENE 432

by Linda Guarino

TITLE - This is the most important part of a manuscript. A reader begins here, and will also finish here if the title does not promise a subject of interest to him/her. A good overall rule is to use the fewest possible words that adequately describe the contents of the paper. But, do not sacrifice words for specific information. For example 'DNA cloning' is a short title, but it is too general. A popular trend in recent years is to publish papers where the title is a complete sentence that summarizes the major conclusion of the manuscript. Personally, I prefer titles that describe the work, not the results.

ABSTRACT - An abstract is a mini version of the paper. It should provide a brief (less than 250 words) summary of the major points of the manuscript. The abstract should state the objectives, describe the methodology used, summarize the results, and state the principle conclusions. The abstract should be written in the past tense, because it refers to work done.

INTRODUCTION - First of all, state the nature and scope of the problem investigated. Review the pertinent literature (NOT NECESSARY FOR THIS CLASS). Describe the method of the investigation. State the principle results. State the principle conclusions suggested by the results. The first two parts should be in present tense, while comments relating to the present study should be in past tense.

METHODS - The methods section should expand upon the description of the methodology that was presented in the abstract. The order of presentation is usually chronological (methods used in initial stages of the study are presented first). However, sometimes it makes more sense to group similar methods into sections, even though they were not used at the same time. Due to space limitations in journals, methods are not usually described in detail if they have previously been published. If a scientist uses a protocol that is identical to one previously described, he/she would state 'The DNA was prepared according the procedure previously described (reference)'. If there were minor differences, he/she would state 'according to the procedure of (ref.) with minor modifications' and then describe the modifications. In this class, you may assume that the class protocol has been published. Therefore you don't need to give the details, but you need to describe the general strategy. For example, you should say 'the DNA was purified by the alkaline lysis procedure as previously described' not 'the DNA was purified as previously described'. In addition to the class protocol, you could also reference the Cloning manual or the Promega manual. The methods section should be written in past tense.

RESULTS - The results section is a presentation of the data. It should not repeat the methods given in the previous section. Each figure should be referred to here. The results section should be written in past tense.

DISCUSSION - The discussion should put the results into perspective. Discuss the results without recapitulating the results section. Show how your results and interpretations agree. State your conclusions clearly, and summarize the evidence for each conclusion. Selection of correct tense is more difficult in the discussion than in the other sections. Your own work should be described in past tense. If reference is made to published work, it should be in present tense.

REFERENCES - Only need to cite the class protocols and any other sources of material...no need for literature review so these are very few.

FIGURES and FIGURE LEGENDS - Present the important data in figure form, raw data if possible for this class. Figure legends begin with a title for the figure. Figures should have complete legends - so that they can be understood without reading the rest of the paper. These may be nested in the paper or placed at the back.

Many students have asked about length. The best rule that I can give you is that it should be long enough to convince me that you have learned something. However, I have a short attention span, and if your paper is very long and verbose, I may lose interest before I decide whether or not you have learned anything.

Suggested reading:

Day, R. A. 1988 How to write and publish a scientific paper, 3rd ed. Oryx Press, Phoenix.

9/6/02

Introduction to Gene Expression

General

The purpose of this class is to provide motivated students with the beginning skills required to apply molecular biology techniques. Protocols and hands-on exercises will teach several techniques, but more importantly, will allow new procedures to be mastered during subsequent research projects. Students will learn how to handle nucleic acids through basic purification and measuring and manipulative procedures. However, comprehensive coverage of the underlying biochemistry of DNA modulatory enzymes, for example, is not possible in the time allotted. Excellent courses exist for such background studies (GENE 431 and 450), and required reading and suggested references will fill those voids. Brief discussions of how applications of molecular biology are used in physiological studies will be held as time permits. Questions are encouraged, also. We will have free time during incubation periods - use these wisely with questions, reading or writing for your notebook or lab report.

The major criticism of this course is that the flow of experiments overlaps. This is life. Experiments proceed too slowly with long incubation times to just sit and wait. It's up to us to keep the purpose of the experiments in our minds (and notebooks!).

This course is designed to help new students study expression of their favorite gene in their favorite tissue. For this class, your favorite genes are actin and glyceraldehyde phosphate dehydrogenase (GAPDH), because they are highly expressed in your favorite tissue (endometrium.) Genes are transcribed into messenger RNAs, so first we'll:

1) Extract RNA from tissue and use it to make a Northern blot. The amount of RNA obtained is measured by sample absorbance at 260nm. To assess RNA quality, the RNA preparation is analyzed on a denaturing gel. This is transferred to a membrane ("Northern blotting") for hybridization with probes for specific mRNAs (actin or GAPDH)..

2) Make a cRNA probe for actin or GAPDH mRNA and hybridize it to the tissue RNA on the Northern blot. The lab boss gives out plasmid clones containing complementary DNAs (cDNA's, synthetic copies of fragments of mRNAs). The best probes are cRNAs. To make them, the circular plasmid DNA is restricted or cut at a specific site with a restriction enzyme. For in vitro transcription, the plasmid and ribonucleotides are combined with a bacteriophage RNA polymerase (SP6 or T7). The polymerase enzyme binds a specific site on the plasmid and transcribes (makes RNA) using the DNA as a template. Either of the two strands of the DNA can be reproduced as cRNA: the top cDNA strand is like mRNA and, if transcribed, is called "sense" cRNA. The sense cRNA is useful as a template for translation. The bottom cDNA strand is complementary to the sense strand and hybridizes to mRNA, as does its transcription product called "antisense" cRNA. We'll only synthesize the antisense cRNA and it will be labeled with Digoxigenin so it will be a probe (detectable reagent) for identifying its

homologous RNA in the tissue RNA samples. After hybridizing the probe to the RNA on the Northern blot and washing the blot, specifically bound probe will be detected with DIG-antibody and chemiluminescent detection reagents.

3) Quantitate estrogen receptor mRNA with RT-PCR. But what if you only have a very small amount of tissue and/or the mRNA you want to study is rare (such as estrogen receptor (ER))? The most sensitive mRNA quantitative technique is quantitative Reverse Transcriptase-polymerase chain reaction (PCR). Reverse transcriptase copies RNA into cDNA. The reverse transcription of tissue RNA will provide cDNA that will be used to amplify a specific target cDNA between two previously designed primers. Since PCR is fickle, the best way to make it quantitative is to use an internal control DNA that competes for the same primers and reagents but can be distinguished (by slightly different size on agarose gels). Therefore, one runs the PCR reactions on an agarose gel and simply looks for the lane with equivalent bands (PCR products) from the internal control and target cDNAs in a titration set of PCR reactions. Since product amounts are the same, starting material must be the same; thus mRNA concentration = internal control concentration, the latter of which is known for that reaction.

4) Subclone beta-globin cDNA to a more useful plasmid vector. But what if the cDNA clone you have is in a plasmid vector that doesn't have SP6 or T7 RNA polymerase sites (many old plasmids don't). You may have to sub-clone the cDNA fragment into your desired vector. The easiest way utilizes PCR to amplify the cDNA. Restriction enzyme sites can be created by synthesizing them on the ends of the primer. This allows easy insertion and ligation of the cDNA and vector. The new plasmid is forced into E. coli cells during transformation, where presence of the plasmid confers a new phenotype to the bacteria: resistance to the antibiotic ampicillin. Clonal colonies are grown and their plasmid DNA prepared (mini-preps) individually to identify the desired clone (by restriction enzyme analysis).

Thus, our different lab exercises all fit together into common techniques utilized in studies of gene expression. KEEP THE FLOW OF LOGIC IN MIND! Don't just come in and mix reagents and shuffle tubes. Think of the molecules and what you want to learn from them. Predict (visualize) the results of your experiments before you perform them.

YOUR SUCCESS DEPENDS ON PREPARATION:

You must read and think through the experiments BEFORE THE LAB to be able to perform and interpret them well. Note that grades are 33% preparation!

Lab 1 9/6/02

Preparing Materials for RNA Work

Beating RNase

You need to read about RNase, a ubiquitous enzyme that efficiently destroys RNA. Primarily, this will serve to make you paranoid and do neurotic things, like wear gloves all the time. Although working with RNA is similar to working with DNA, many RNA experiments fail miserably because of RNase, so know this enemy!

In biochemistry, RNase is the model of an enzyme that will not die: not in an autoclave or even after dehydration (by alcohol, etc.). As soon as it returns to a water environment between room temperature (R.T.) and 37°C, it chews again. It is an enzyme of all living things and is important in keeping RNA turnover high so cells don't choke on RNA and so new expression of genes tightly regulates cell function.

The best way to beat RNase is to avoid it. Work with the cleanest reagents and lab-ware. Things that aren't handled by living thing are generally RNase-free; e.g. paper towels. Test tubes and pipettes don't have to be sterile but should be used from freshly opened packages. Then protect packages from dust and fingers by resealing packages and storing in cabinets. Glassware is reserved similarly: wrapped and stored away from general use. Equipment like Pipetmen and Gel apparati for RNA are reserved for this use and are NOT USED WITH RNase!

Solutions are made with water of the highest purity. Dry chemicals are shaken out of containers: residual amounts are discarded. Nothing dirty is introduced into chemical stocks: solutions or powders.

All solutions are treated with 0.1% diethyl pyrocarbonate (DEPC). This oily liquid is added. The solution is shaken vigorously until foamy (aerobic workout). The solution is incubated 37°C overnight to allow the DEPC to covalently attack RNase. The solution is then autoclaved to destroy DEPC (which also attacks RNA) and to sterilize to prevent growth of undesirables. Exceptions to this solution preparation protocol are 20% SDS (nothing grows in this) and Tris solutions (which DEPC attacks, too). NOTE: DEPC treatment can only correct a low level of RNase contamination! You must start clean!!!

Today's Exercise

Apply benchcote and work with gloves on and tubes/racks on top of diapers.

A. Pack tips: 1 blue box and 2 yellow boxes per group

1. Pour clean blue tips onto a clean surface (paper towel or diaper).
2. With new gloves, pack tips.
3. Write name on box & protect it!
4. Repeat with yellow tips: fill 2 boxes.

B. Practice pipetting

1. Read instructions for pipetting in Appendix.
2. Tare a 1.5 ml test tube on the balance.
4. Weigh 1000 ul of water two times.

5. Repeat with Isopropanol.
6. Determine the densities of these liquids.
7. Measure the volumes of the unknown samples provided in the 1.5ml test tubes.
8. Check your results with an instructor.

C. Each person should test tap water, distilled and DEPC-treated water for RNase:

1. Label 4 RNase-free 1.5 ml tubes. Pipet 4 ul of either tap, distilled or DEPC H₂O into each (two tubes get DEPC-H₂O).
2. Pipet 1ul test RNA into each tube.
3. Put all but one DEPC-H₂O tube in 37°C block for 1 h. (The lone DEPC-H₂O tube should be kept on ice during the 1 h incubation.)
4. Store all of the tubes at -80°C until Lab 4.

D. Make 1 li DEPC-H₂O and 1 li of 20XSSC per person

1. In 1 liter bottle add nanopure H₂O to 1 liter level for DEPC-H₂O. For 20X SSC, add 175 g NaCl and 88 g Na citrate to a 1 li bottle and dissolve in nanopure H₂O.
2. Add 1ml DEPC per liter.
3. Shake till foamy for 10 sec.
4. Put in 37°C incubator O/N to allow DEPC to work optimally.

Lab 2 9/13/02

RNA Extraction

[Autoclave the DEPC-H₂O to destroy DEPC and prevent any growth in solutions that might introduce RNase. NOTE on Autoclaving: Need 35 to 40 min. sterilization time for 1 liter. 20 min. for 500ml. Use "liquid" cycle and keep caps loose]

You know how to fight RNase to keep materials clean. GUESS WHAT! RNase is in all living systems including the one from which you'll purify RNA. So all RNA preparers begin with the realization that their worst enzyme enemy is present in the sample. In the cell, RNA is compartmentalized away from RNase so many tissues are OK for harvesting for RNA if kept cool 2-6 h after collection (of course, faster may be better). But freezing breaks intra-cellular membranes, mixing RNA with RNase. Therefore, fresh tissues are kept cool while mincing and

weighing, then are put in a 1.5 ml polypropylene tube snap frozen in liquid N₂. They are stored at -80°C. They may store well for 6 months but usually not for 1-2 years. This is dependent on them never thawing, too. So the TWO MAIN POINTS about tissue collection are to SNAP FREEZE and KEEP at -80°C until use within 1 year. NOTE: You can't snap freeze things much bigger than 0.5 cm³. I mince to about 5 mm or less. Tissues vary with RNase content and amount of connective tissue present, so RNA yields vary in quality and amount. RNA extraction from cultured cells results in very high quality RNA, usually.

RNA Extraction from tissue with Boehringer Mannheim TriPure reagent (contains phenol! see NOTE 1 below)

Each student will do 2 RNA preps (one from endometrium, one from spleen). Label all tubes needed NOW!

1. Homogenize 0.5 mg tissue (frozen or fresh) in 5 ml room temperature ("RT") Tripure solution in a 50 ml polypropylene tube. Use three 15 sec bursts at 70% power. Rinse probe in tripure (do a mock homogenization with Tripure and no tissue) between dissimilar samples.
2. Incubate RT 5min. During this time, transfer the contents equally into 4 - 1.5 ml tubes.
3. Add 250 ul chloroform using a P-1000. Mix by vortexing or shaking vigorously 15 sec.
4. Incubate RT 5 min.
5. Centrifuge 15 min at 10,000 rpm at RT or 4°C in a microfuge.
6. Transfer upper phase to four clean 1.5 ml tubes with transfer pipet. AVOID THE INTERFACE!!!! Discard lower phase and interface in phenol waste container.
7. Precipitate RNA by adding an equal volume of isopropanol. Mix by inverting tube. Incubate RT 5 min
8. Centrifuge at 10,000 rpm for 10 min at RT.
9. Wash pellet in 75% EtOH (make 10 ml with 100% EtOH and DEPC H₂O). This means to discard the supernatant, add the supernatant volume of wash (75% EtOH), vortex, microfuge 5 min, and discard supernatant. The purpose is to wash salts out of the RNA pellet, which should not dissolve during the procedure.
10. Air dry pellet briefly after spin. You can wipe the sides of the tubes with Kimwipes, but stay away from the pellets! Do not dry totally or you will not be able to solubilize RNA easily!!!
11. Store pellet at -80°C.

NOTE 1: Tripure has phenol and guanidine salts in it...both are caustic and burn skin!!! Be careful! Wear safety glasses!!!

NOTE 2: CHCl₃ (Chloroform) dissolves things like styrofoam and polystyrene - use glass graduated pipets and polypropylene 15 & 50 ml tubes.

BEFORE YOU LEAVE, CLEAN UP AND RECORD ACTIVITIES IN NOTEBOOK!!!

NOTE 3: Record observations in notebook!

Examples: 1) Lysate in step 2 was viscous!

2) Tube #2 fell and was lost.

3) RNA pellet #5 took 20 min. to dissolve, while #1 took only 1 min.

Lab 3 9/20

Analyzing Extracted RNA by Absorbances; In Vitro Transcription of cRNA Probes

A. Solubilize RNA samples from tissue and measure Absorbance at 260 nm and 280 nm

Absorbance measures of DNA & RNA at 260 nm are used to estimate concentrations of nucleic acids. An Absorbance of 1.0 for solution of double-stranded (ds) DNA has =50 ug/ml while RNA has $A_{260} = 40$ ug/ml and single-stranded (ss) DNA $A_{260} = 37$ ug/ml. An unknown sample of RNA can be measured for A_{260} and $[RNA] = A_{260} \times \text{dilution factor} \times 40$

The ratio of A_{260}/A_{280} is an indication of the purity of the nucleic acid. The ratio for pure aqueous DNA is 1.8 while for RNA it is 2.0. Protein, phenol, EtOH and other things often lower these ratios because they absorb at A_{280} .

1. Dissolve the four similar pellets each in 25 ul 1 mM Na citrate Buffer/pH 6.4 or TE buffer (10 mM Tris, 1 mM EDTA pH8).

(Heat in 70°C block and vortex hard and repeatedly over 15 minutes.) Pool so that you have a 100 ul sample for each RNA prep.

{RNA STORAGE: Store at 4°C during sample use (this class). For storage over 1 week, can store at -80°C. For longer storage, add 3 volumes of ethanol and store at -80°C.}

2. Add 0.5 ml of DEPC-H₂O to seven 1.5 ml tubes.

3. Label one "Blank" and the others (duplicates) after the samples: the two RNA samples and a positive control: 10mg/ml salmon sperm DNA.

4. Add 2 ul aliquots of samples to @ tube except "Blank".

5. Use micro UV-transparent disposable cuvettes and rubber or plastic transfer pipettes. Blank the machine to read 0 absorbance at A_{260} nm with the "Blank" in a cuvette. Then repeat with dilute samples in cuvettes. Repeat the procedure for measuring the A_{280} of samples.

6. Estimate $[RNA]$ (ug/ml) = $A_{260} \times \text{diln. Factor} \times 40$

= $A_{260} \times 250 \times 40$

Therefore: [RNA] (mg/ml) = A260 * 10 (= ug/ul, too!)

7. Pipet 32 ug RNA into a clean 1.5 ml tube for each RNA prep. We want 32 ug aliquots of RNA for running replicate 8 ug samples on a Northern gel. If volume is less than or equal to 15 ul, store "as is" at -80oC. If volume is greater than 15 ul, precipitate the RNA by adding 3 volumes of 100% EtOH and 0.1 volumes of 3 M NaAc/pH 5.2. Vortex and store at -80oC.

8. To the rest of the RNA preps, add 3 vol 100% EtOH and store at -80oC. (This is a good way to store RNA without degradation for years.)

B. In vitro transcription - DO THIS FIRST!

NOTE : There are 3 common types of nucleotide probes: DNA oligonucleotides (ss), cDNA (ds) and cRNA (ss). For many applications, cRNA probes are superior over:

1. end-labelled oligonucleotide probes because they are:

a. longer (and therefore carry more label and have higher hybridization specificity)

b. uniformly labelled throughout (so they carry more label)

2. nick-translated or random-primed cDNA probes, because they only have the desired probe strand, not the other "sense" strand that increases background.

In addition, the binding of RNA:RNA hybrids is stronger than that of DNA:DNA hybrids.

REMEMBER:

1.the cDNAs are synthetic cloned fragments of the mRNAs

and

2. Knowing the information on the plasmid maps (See appendix) is critical to designing the probes (e.g. knowing what enzyme to linearize the plasmid with, which enzyme to transcribe with, and which strand (sense or antisense) is generated.

Circular plasmids must be linearized with a restriction enzyme to generate DNA templates suitable for in vitro transcription. You will use SP6 or T7 RNA polymerase to transcribe antisense cRNAs for actin and GAPDH mRNAs and 18S rRNA. Ambion's "pTRI-_____" constructs are foolproof: already linearized, and have all the RNA polymerase sites on the side of the cDNA so as to make only antisense transcripts. (REMEMBER THAT RNA IS SINGLE-STRANDED!!!)

ANSWER THESE and all other PROTOCOL QUESTIONS IN YOUR NOTEBOOK

Q1.: If we wanted to probe a Northern blot with cRNA from poPR77A, which restriction enzyme and RNA polymerase would we use with that plasmid? (see map).

Q2.: If we wanted to do in vitro translation with cRNA from poPR77A, which restriction enzyme and RNA polymerase would we use?

[In vitro transcription kits can be obtained from various sources. Ambion's "Maxiscript" is OK and is the basis for the following reaction (one per group).]

A. In vitro Transcription using the Roche DIG-Labeling kit.

Each student should set up one in vitro transcription reaction for DIGOXIGENIN-labeling antisense 18S, GAPDH, or actin cRNA probes:

1. Thaw components at room temperature (RT) then store on ice.

EXCEPTION: RNasin and RNA Polymerase, like all enzymes, stay at -20oC always!

2. For sense cRNA add components from kit, in order, to a 0.5 ml tube at RT.

12 ul DEPC-H₂O

2 ul 10X Transcription Buffer

1 ul RNasin

2ul 10 mM rATP, rCTP, rGTP, and 3.5 mM DIG-11-UTP

2 ul linearized DNA template

1 ul SP6 or T7 RNA Polymerase

3. Mix by pipetting up and down...gently! No bubbles.

4. Flash spin in a microfuge

5. Incubate 37oC for 1h

6. Add 1 ul RNase-free DNase

7. Incubate 15 min at 37oC

8. Save 5 ul of each of the transcription reactions in 1.5 ml tubes for analysis on a urea 5% acrylamide short fat sequencing gel. Store these tubes and the original reaction tubes at -80oC.

9. For short fat acrylamide gels in next lab, wash 1 short and 1 long plate with soap and water and a 250 ml Erlenmeyer flask or beaker. Soak in 1 M NaOH overnight (O/N).

In Vitro Transcribed RNA (and RNase test) analysis on urea/acrylamide gel

A. Analyze in vitro transcription products on a 8M urea, 5% acrylamide gel called a "probe test gel or short, fat sequencing gel." These denaturing acrylamide gels are especially suitable for analysis of small (<400 base) DNAs and RNAs.

1. Make a probe test gel. (See Appendix recipe and plate setup for vertical gel.)
2. Add 15 ul of deionized formamide loading dye to your 5 ul cRNA aliquots as well as to your RNA samples from the first lab (RNase test). Similarly, prepare a sample of RNA Century (Ambion) markers. Leave the 15 ul aliquots of the cRNA reactions at -80oC!
3. Heat 68oC for 5 min.
4. Cool on ice.
5. Flush wells free of urea with 1X TBE in needle & syringe.
6. Load with elongated gel loading tips carefully! in the bottom of wells.
7. Run at 35 mamps for 1 h.*
8. Disassemble gel plates.
9. Stain the gel with ethidium bromide by soaking in 2 ug/ml EtBr for 5 min. Use gentle agitation.
10. Destain by soaking in d H2O for 5 min. with agitation.
11. Photograph the gel on a UV light box. Label the photograph completely (see Lab Rules A4) and tape it in your notebook.
12. Compare migration distances and brightnesses of bands with those in RNA marker lane. Note the migration of the tracking dyes, too (see table in APPENDIX) (Xylene cyanol is light blue & slow, while bromphenol blue is dark blue and fast). Mark the dye migration positions on the photograph so you could rerun the gel exactly if you wanted to.
13. For the next lab, treat a 250 ml Erlenmeyer flask or beaker and a mid-size horizontal gel apparatus with 1 M NaOH. Leave these to soak until next time.
14. Do calculations for Lab 5 step A6. Dilute a small amount of plasmid DNA template for in vitro transcription to 10 ng/ul. Store at -20oC.

*During the gel run, discuss DNA structure using human nucleotide models:(answer all questions in your notebook!)

- a. Make a single-stranded six base random sequence and identify 5' and 3' ends. What types of bonds exist between the bases?

- b. What is the chance that a specific 6 base sequence will occur randomly?
- c. Reverse the sequence polarity. What bonds did you have to break?
- d. Make a complementary strand of DNA. How are the strands oriented to each other? How is this strand's sequence related to the initial strand? What types of bonds are between the strands?
- e. Make an EcoRI restriction enzyme site. What bonds does the enzyme cut? What makes the ends "sticky"? Do the ends have 5' or 3' overhangs?

Lab 5 10/4/02

Northern Gels (NorthernMax (Ambion) protocol) and Blotting

Be clean - protect apparatus from RNase contamination- clean diapers!

All DEPC reagents

A. Recover 32 ug total cellular RNA samples from the endometrial and liver extracts that you made (2 per student).

For each RNA prep, make a 32 ug sample to provide duplicate samples for two lanes. If samples from C7 of Lab 3 are precipitated start at 1 and continue. If not, start at step 6.

1. Spin 10 min at 4°C at 10,000X g
2. Discard supernatant by decanting
3. Dry sides of tubes with Kimwipe and remove residual ethanol from pellet with yellow tip if needed.
4. Air dry pellets 5 min.
5. Dissolve pellet in 15 ul 1 mM Na citrate at 68°C with vortexing.
6. If your sample was not precipitated, bring it up to 15 ul with 1 mM Na citrate. Add 45 ul Northern Sample Loading Dye and 6 ul of 0.1 mg/ml EtBr to each sample. These will be loaded in two lanes as described below. ALSO, prepare 10 ng samples of linearized plasmids with cDNAs for 18S rRNA, GAPDH, and actin (diluted to 10 ng/ul in Lab 4 step 14) in 5 ul 1 mM Na citrate + 15 ul dye. Add 2 ul of 0.1 mg/ml EtBr to each sample. Lastly, prepare a 15 ul sample of mouse liver control RNA to be split into two lanes during loading.

Q: What is the concentration of the control RNA? How much is in each lane?

7. Heat 68°C for 10 min.
8. Chill on ice

B. Prepare & Run gel. 2 people per gel: one student load the top row, one the bottom row of wells

START THIS FIRST OR SIMULTANEOUSLY WITH YOUR RNA SAMPLE PREPS (above)!

1. Rinse gel rig and beaker/flask well with house-distilled water. Melt agarose (0.8 g) in 72 ml DEPC-H₂O in an RNase-Free glass bottle or beaker. (Bring to a boil in microwave oven and mix by swirling: repeat 2 to 3 times).

2. Cool to 70°C.

3. In a fume hood, add 8 ml 10X Denaturing Gel buffer (formaldehyde and MOPS/pH 7.0, NaAc, and EDTA) and pour into RNase-free gel mold with the ends taped. Use two thin combs.

4. Load samples onto the gel under 1X Gel Running Buffer (dilute the 10X stock from the kit with your DEPC-H₂O bottle, cover the gel with buffer to about 0.5 cm depth). LOADING ORDER[Skip spaces = "X"]

Millenium RNA markers, Positive control RNA , student1 Endometrial RNA, student1 Liver RNA, pTRI- actin, pTRI-GAPDH, pTRI-18S rRNA, student2 Endometrial RNA, student2 Liver RNA plasmid.

5. Run at 100 volts until dye front reaches bottom or sufficient separation occurs (1 to 1.5 h); can peek at gel progress with hand-held short wave UV lamp. Prepare materials for blotting during this time!!!!!!!!!!!!

6. Take photo on UV box alongside a fluorescent ruler.

NOTE: MARK DYE POSITIONS ON GEL PHOTOGRAPHS. Label all photos completely! Name, date, identity!!! Number lanes on photo then describe them in your lab notebook.

C. Northern transfer to nylon membrane

1. Cut wicks, blotting papers, and nylon membrane wet in transfer buffer as directed.

2. Soak gel in 10X SSC for 15 minutes with gentle agitation.

3. Assemble an upward capillary transfer as instructed in the diagram. You can use the rig you ran the gel in to do the transfer to nylon. Allow the transfer to continue with 500 ml of 10X SSC until the next lab.

Lab 6 10/11

Northern Blot hybridization

A. UV cross-link RNA to Northern blot

1. Remove all papers from the Northern transfer but keep the gel and blot together. , mark well positions on the blot with a sharp pencil or a black Sharpie marker. Also write initials and date. Put these marks on filter's back. Keep the blot RNA-side-up during subsequent handling.
3. Rinse filter for 30 sec. in 2X SSC with vigorous agitation.
4. Put blot on plastic wrap and on UV box. On the side of the blot, mark positions of 28S and 18S rRNAs and RNA markers. Also, mark places you will cut the blot in the future (to hybridize with different probes), e.g., on the blank lane #6. You can take a picture to confirm that the RNA transfer was good. Look at the gel on the UV box. Did all of the RNA transfer out?
5. Place wet blot on top of Whatman paper saturated with 2XSSC - all on top of plastic wrap.
6. UV crosslink RNA to nylon (use Stratalinker in energy mode: 120,000 (or 1,200,000 ?) uJoules).
7. Cut the blot into pieces to be probed with 18S rRNA and GAPDH and actin probes.

B. Prehybridization & Hybridization

Since the nylon membrane likes to bind things, background sites are blocked (bound) with non-specific DNA and protein. Usually, sheared salmon sperm DNA is used in prehybe to block these sites.

1. Warm the Ambion Ultrahybe hybridization solution to 65oC. Swirl to dissolve precipitates.
2. Make three plastic bags from a tube with the heat sealer. Double seals are a good idea. The bags should be 2 cm longer and wider than blots. Leave one end open and insert the dry blot . Wet it with 2X SSC. Pour 2X SSC out.
3. Add 5 to 10 ml Hybridization solution and seal bag. Note that bags are harder to seal when they contain the fluid, so you may have to turn up the sealing time on the heat sealer to get a good seal.
4. Incubate 68oC for 30 min. During this time, calculate how much of each probe to add to a fresh 10 mls of Ultrahybe to reach 10 to 20 ng/ml (about 0.1 nM).
5. Heat cRNA probe at 94oC for 10 min - chill on ice.
6. Cut off a corner of the bag.
7. Discard prehybe buffer. Add Ultrahybe containing the appropriate probe to each blot.
8. Reseal bag with heat sealer
9. Incubate 65oC overnight or over weekend.
10. Predict Blot results

Based on your blot photo and what you know about actin mRNA (2100 bases) and GAPDH mRNA (1400 bases), draw your expected hybridization results in your notebook. Use the 28S & 18S rRNA positions as markers. For the mouse, 28S rRNA is 4718 bases and 18S rRNA is 1847 bases. There should be 2 to 6 pg of GAPDH mRNA in 5 ug of the mouse liver RNA + control.

Q: How much RNA is in each rRNA band?

C. Assess quality of probes.

1. Make 4 serial 1:9 dilutions of each probe: Pipet 1 undiluted probe into a tube with 9 ul TE and mix. This is a 1:10 dilution. Repeat the dilution 3 times; so have 1:10, 1:100, 1:1000 and 1:10,000 dilutions.
2. On three 3 by 10 cm strips of nylon membrane, mark membrane with sharp pencil or black Sharpie pen "-1", "-2", "-3", "-4" at 2 cm intervals. Labelled one for each probe. Add initials and date.
3. Dot 1 ul of appropriate probe dilutions under labels. UV Cross-link RNA to membrane as you did for the Northern blot.
4. Air dry and store in a clean place. (You'll develop the dot blot along with the Northern blot in the next lab.)

Lab7 10/18

Northern Blot Washing and Development; Making cDNA by Reverse Transcription

A. Northern blot washing and development

After overnight hybridization, probe is maximally bound to specific sequences. It is also present on some non-specific sites. By reducing [salt], mainly in the form of SSC, hybridizations are tested for stringency. Usually temperature is increased as well so that probe-binding is specific for the target of interest.

KEEP BLOTS WET DURING THESE PROCEDURES or you'll generate a lot of artifacts.

1. Cut off corner of hybridization bag. Discard hybe solution.
2. Cut bag open and move blot to a clean tupperware container. Put all 3 blots in the same container. Seal the lids during washes so solutions don't spill onto the shakers!!!
3. Wash the 3 blots in 100 mls of 42oC 2x SSC with shaking for 15 min.
4. Discard wash and repeat.

5. Wash in 100 mls 0.5 X wash solution [Maleic acid buffer (1X = 0.1 M maleic acid, 0.15 M NaCl, pH 7.5) with 0.3% Tween-20] at 65°C for 15 min. Repeat once.

6. Use CDP-Star detection system at RT:

a. Add in the probe dot blot from Lab 6C at this step. Equilibrate membranes in washing buffer 1 min:

b. Allow chemiluminescent substrate to come to RT.

c. In freshly washed tupperware, block the membranes for 30 min in 20 ml block solution (1% (w/v) blocking reagent in maleic acid buffer without Tween). During gentle shaking, membranes should move independently from each other.

d. Discard block and incubate in Anti-DIG-Alkaline Phosphatase antibody solution (1:20,000 diluted in block solution) for 30 min. Use a minimal volume (20 ml) in a small clean container like a yellow tip box lid. During gentle shaking, membranes should move independently from each other.

e. Discard the antibody solution and wash twice in washing buffer for 15 min each time.

f. Discard wash and equilibrate membrane in detection buffer for 2 min.

g. Pipet 1 ml of CDP-star detection reagent diluted 1:100 in detection buffer onto each blot and cover with plastic wrap. Incubate 5 min, then pour off excess reagent.

7. Wrap blot in saran wrap. To keep the blot wet and the film dry, double fold the plastic wrap and tuck all edges under the blot. As always, RNA side up!

8. Place in cassette.

9. Go to the dark room and lay a piece of film on the blot. Bend the lower right corner of the film. Close the cassette. Make sure you fold the flaps so it is light tight!

10. Place cassette at 37°C or room temperature. Develop the film in 30 min. If desired, place new film on and expose longer...O/N?.

B. Making cDNA by Reverse transcription

To produce cDNA for cloning, mRNA is reverse transcribed. Typically, an oligo dT primer is annealed to the poly A tails of the mRNA's. The Reverse Transcriptase (RT), the enzyme retroviruses use to copy their RNA genomes, copies the RNA into cDNA. This reaction is used for first strand cDNA synthesis, the first step in formation of all cDNA libraries, and is the basis for true molecular cloning.

Each person will perform one RT synthesis of cDNA on their favorite RNA sample.

1. Spin down stock RNA sample after adding 1/10 vol 3M NaAc and mixing.

Dissolve in a volume of 1 mM Na citrate to make solution 1 ug/ul.

(estimate from absorbance measurements). Measure A260. Pipet 10 ug into clean tube and bring up to 25 ul volume with 1 mM Na citrate.

2. Combine in a 1.5 ml tube at RT, in order:

5.0 ul DEPC-H₂O

5 ul 5X AMV RT Buffer

1 ul 100 mM DTT

2 ul dT17 primer

5 ul RNA (2 ug)

3. Heat 68°C 5 min.

4. Let cool to RT slowly.

5. Add 5.0 ul 5 mM dNTP

1 ul RNasin

1 ul AMV RT

6. Incubate 37°C, 1 h.

7. Heat at 94°C for 5 min. to kill RT.

8. Store at -20°C.

Lab 8 10/25

Analysis of Blots; Quantitative RT-PCR

A. Northern Blot Analysis

1. Develop the autoradiograph in the film processor. Label films with exposure date, time and index to your notebook. Align with the blot and mark the well, 28S & 18S rRNA positions on the film. What are the positions of the hybridized bands? Are the bands more intense in RNA samples from one tissue compared to the other?

2. Qualitatively assess the blot results:

a. Is exposure optimal?

b. How many bands are evident? Are they in the expected tissues?

c. What is the size of the darkest band? To do this measure migration distances for the band on the Xray film, and for 28S (4800 bases) and 18S (1800 bases) rRNAs on the EtBr stained gel photo using UV ruler as a guide. On graph paper, plot log base length against migration distance for the rRNAs. Find log bases from the plot, using the migration distance of the band of interest. Find antilog to get number of bases. If RNA markers show up, you can use those instead of or in addition to the 18 and 28S rRNAs.

3. Quantitate blots –

a. Can use densitometry on the Xray film. Need a good scanner and analytical software such as BiImage IQ.

b. Many machines are being developed for direct scanning of blots. These are very powerful because they avoid the limitations of film.

B. Quantitative PCR analysis of ER mRNA in an RNA sample

NOTE: Be clean (Don't introduce exogenous DNA) USE AEROSOL BARRIER TIPS! WEAR GLOVES!

PCR amplification is very useful in detecting small amounts of nucleic acids. Since the amplification can be described mathematically in theory, then it should be possible to calculate back to the original concentration of template. Problems arise because the exponential amplification is seldom realized. To reduce variability and gain quantitative ability, PCR reactions are done with the fewest possible cycles under limiting reagent conditions. An internal competitive template, with the same primer/sites on a shorter piece of DNA, can be added in increasing amounts to a PCR reaction. In the reaction with equivalent products: large from the template of interest and small from the internal competitor; the starting amounts of template are equivalent. So by this PCR titration with competitor template and gel analysis with EtBr staining, templates of interest can be quantitated. Quantitative PCR is very well explained in Clontech's MIMIC manual.

How do you get template DNA? The quantitative PCR can be tested on linearized plasmid DNA. But to use PCR to estimate [specific mRNA] in an RNA sample, it is first reverse transcribed to cDNA as you did in the last lab.

Each person will do a set of five quantitative PCR reactions on one RT-cDNA sample, as well as (+) and (-) PCR controls.

1. At RT, combine (in order) 8X amounts of the first five reagents to make a master mix. Add 23 ul of master mix to seven 200 ul thin-walled tubes, then add the template and internal control DNAs to each individual tube.

Master mix: 17.5 ul sterile H₂O X8 = 140 ul

2.5 ul 10X Taq Buffer containing Mg⁺⁺ X8 = 20ul

1 ul 5 mM dNTPs X8 = 8 ul

1 ul primer A (ERPCR1, 10 ng/ul) X8 = 8 ul

1 ul primer B (ERPCR2, 10 ng/ul) X8 = 8 ul

2. Do serial dilutions (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4}) in sterile water of the provided internal control DNA (linearized poER8short, 100 amol/ul). Add DNAs to your tubes:

TUBE #1 1 ul RT-cDNA and 1 ul 10^{-4} internal control

TUBE #2 1 ul RT-cDNA and 1 ul 10^{-3} internal control

TUBE #3 1 ul RT-cDNA and 1 ul 10^{-2} internal control

TUBE #4 1 ul RT-cDNA and 1 ul 10^{-1} internal control

TUBE #5 1 ul RT-cDNA and 1 ul undiluted internal control

TUBE #6 1 ul positive control plasmid (poER8, linearized, 1 amol/ul) and 1 ul H₂O

TUBE #7 2 ul H₂O [negative (no template) control]

All should have 25 ul final reaction volumes.

Note: PCR primer sequences are:

ERPCR1 - (5') agcccagcggctacaggtgc

ERPCR2 - (5') gcaggcctggcagctcttcctct

3. Program the PCR machine to do 40 cycles of:

94°C for 30 sec: strand separation

50°C for 30 sec: primer annealing

72°C for 1 min: polymerization

4. Put your 7 PCR tubes in the PCR machine. Add 0.25 ul Taq DNA Polymerase with a P-2 Pipetteman and tips during a 94°C hot start (make machine hold at 95°C for 2 min then add enzyme. You can also do this in a hot block or bath. This "hot start" prevents lots of nonspecific product from primers annealing non-specifically with the template at RT.) Run the cycling program after enzyme addition.

5. Make one 2% agarose gel for each two students: 125 mls of 2% agarose gel + 0.5 ug/ml EtBr in 1X TAE; use 2 thin combs for each gel.

6.. Store PCR reactions at -20°C. Store gel in the gel mold with the comb, wrapped in saran wrap with a little buffer and in a sealed tupperware container or pyrex dish at 4°C.

Lab 9 11/1

Quantitative RT-PCR Analysis; PCR Subcloning

A. Quantitative PCR Analysis

1. Add 2.5 ul 10x DNA dye to each 25 ul of PCR reaction. Mix by pipetting up and down.
2. Load the entire samples (or as much as possible in the well) on the 2% gel made in previous lab. Also load pGEM DNA markers (2ug = 20 ul).
3. Run at 120 V for 1 h.
4. Photograph.
5. Estimate
 - a. Sizes of products in bp.
 - b. [target cDNA] in RT-cDNA reaction.

NOTE: The ratio of the target cDNA (315 bp) to the internal control (249 bp) is the critical endpoint. Find the reaction where the two bands are nearest to being equivalent in brightness of EtBr staining, and that is the reaction that started out with equal amounts of target mRNA and internal control. Since you know how much of the latter you added, you know how much of the target was in the sample. You can now determine the concentration of mRNA in your original RNA prep. (You can assume the RT reaction made one cDNA copy of each mRNA present.)

To make this technique amenable to comparing mRNA levels in a large sample set, a single internal standard concentration equal to the average target concentration is used with each sample. Radioactive dNTP's can be used to label the products so their ratios can be quantitated. There are machines that quantitate UV fluorescence directly from gels, too.

NOTES on INTERPRETING AGAROSE GEL INFORMATION:

By comparing the bands in plasmid lanes with DNA standards, one can describe

- a. The molecular size of the fragment, and
- b. The [DNA] of each fragment

For (a), compare migration distance to that of DNA standard fragments - estimate bp size.

For (b), compare brightness of bands to those of DNA fragments - estimate ng. Divide by the amount of sample loaded to get [DNA]. This is often more reliable quantitation than A260

measures!!!

NOTE: MARK DYE POSITIONS ON GEL PHOTOGRAPHS. Label all photos completely!
Name, date, identity!!!

B. PCR sub-cloning (see cartoon following)

One of the most useful applications of the polymerase chain reaction (PCR) is easy subcloning. Subcloning, taking a piece of cloned DNA and transferring to a new vector, used to be limited due to the scarcity of naturally occurring restriction enzyme sites. One was forced to choose DNAs with restriction sites, often these were for weird, expensive, and inefficient restriction enzymes. With PCR, restriction enzyme sites can be engineered on any DNA by adding the desired sites to the ends of the primers. Note that 5' and 3' primers must have different sites to prevent them from annealing. After restricting the PCR product, sites are different on the ends and can only enter a similarly restricted vector in one orientation: therefore, this is "Directional cloning."

Although PCR can be temperamental, subcloning fragments less than 1000 bp is, dare I say, fairly easy.

Here, we'll subclone our beta globin cDNA into a different plasmid vector. The old vector was pBluescript (Stratagene); the new pET-5a (Promega).

Q: What specific advantages does the new vector have (see Promega catalog or protocol book)? List these in your notebook.

Do 1 PCR reaction per person. Be clean (Don't introduce exogenous DNA): USE AEROSOL BARRIER TIPS! WEAR GLOVES!

1. Combine, in order, at RT in a 200 ul thin-walled PCR tube

17.5 ul H₂O

2.5 ul 10X Taq Buffer containing MgCl₂

1 ul 5 mM dNTPs

1 ul primer Xba-GLOB (0.25 ug/ul)

1 ul primer GLOB-R1 (0.25 ug/ul)

1 ul linearized pGLOBcdsonly

0.5 ul Taq DNA Polymerase

Note: primer sequences are:

Xba-GLOB (5') gctctagatgctggtgtctacccatgg

GLOB-R1 (5') gcgaattctgaagttctcaggatccacg

2. Mix by pipetting up and down

3. Program a PCR machine for 35 cycles of:

94oC for 30 sec: strand separation

50oC for 30 sec: primer annealing

72oC for 1 min: polymerization

4. When the PCR is complete, the reactions will be stored at -20oC until the next lab session.

Lab 10 11/8

DNA Restriction and Ligation

A. DNA Restriction and purification on gel

Ligations are the weak point in most cloning procedures. Here we are set for success by using "sticky ends" restriction sites with 4 base overhangs that like to pair up. We'll also do the ligation directly in low melting agarose to minimize loss of DNA during purification.

NOTE: Restriction Enzyme cuts usually contain 1-2 ug DNA and 5-10 units of enzyme in 20 ul. Working buffer strength is always 1X. Use the buffer supplied with the enzyme if it is a single enzyme cut. If using two enzymes (as here), use a buffer compatible to both! Which one should you use????? There is a table in the Promega book that will help you choose.

1. Add 25 ul of TE to the PCR reaction and transfer the 50 ul to a 500 ul microfuge tube.

2. Extract with Phenol/Chloroform/IAA (25:24:1) pH 8. (This means to add an equal volume of Phenol/Chloroform/IAA, vortex to make an emulsion, microfuge 2 min., then transfer the upper aqueous phase that contains the DNA to a clean, labeled tube.)

3. Extract with Chloroform/IAA (24:1)

4. Set up a restriction digest for the PCR product:

16 ul PCR product (insert)

2 ul 10X ?????? Buffer

1ul EcoR1

1 ul Xba I

20 ul TOTAL volume

5. AT THE SAME TIME, RESTRICT 0.5 ug of the vector pET-5a (see map in Promega book) as in 4. Check calculations for the reaction with the instructor.

6. Incubate at 37oC for 1 h.

7. Make a 0.8% low melt agarose gel + EtBr. Raise comb by adding small squares of tape. These gels are like soft jello - Hard to handle!! Be careful removing combs, etc.

8. Add 2 ul 10X Loading dye to the digest reaction. Load it and 1 ug Lambda HindIII EcoR I DNA markers on the gel. Run at 100 V for 30 min.

9. Cut out bands and remove excess agarose while visualizing under Long wave UV light. Wear goggles!

B. DNA LIGATION

1. Melt DNA + agarose 70oC, 10 min. Then keep tube at 37oC until through pipetting.

2. Label two ligation tubes. Put them at 37oC. Add 1 ul vector DNA in gel to each. To the ligation tube, add 3 ul insert DNA in gel and 5 ul H2O. To the control tube add 8 ul H2O. Keep these at 37oC for at least 2-3 min and until the master mix is added.

3. Make a Master mix composed of:

2 ul 10X Ligase Buffer x 3 = 6 ul

8 ul H2O x 3 = 24 ul

1 ul T4DNA Ligase x3 = 3 ul

11 ul per reaction

Add 11 ul ice-cold mix to each ligation tube. Finger flick the tube immediately and slam on ice. Reactions will gel while ligation occurs.

4. Incubate 15oC, O/N. This is done in a hot block in a 4oC room.

Lab 11 11/15

Transformation of Bacteria

"Bacterial transformation" relates to the change of bacterial phenotype by introducing a

plasmid containing an antibiotic resistance gene. "Competent cells" are made receptive to plasmids by making their membranes permeable with calcium treatment. Plasmids adhere to cells, enter on heat shock, and cells are selected for antibiotic resistance on plates after a 1 h recovery period in broth. NOTE: All waste contaminated with E. coli must be killed with bleach or discarded in "BIOHAZARD BAGS," which are autoclaved prior to disposal.

Each group does three transformations: one from the vector only control ligation and one from the ligation that has DNA insert too, as well as a positive control for transformation: 10 ng of circular plasmid. For the last, use any plasmid.

1. Thaw cells on ice (20 min.)
2. Pipet 100 ul into a cool 1.5 ml tubes: 1 for each ligation (vector only and vector+insert), 1 for the positive control
3. Melt ligations at 70oC, 10 min.
4. Cool ligations to 37oC in block, at least 2 min.
5. Add 1 ul ligation to cells; mix by pipetting up and down.
6. Incubate on ice 30 min. Mix every 10 min. by tapping th tube gently.
7. Heat shock 42oC, 45 sec (Be exact here!). No shaking.
8. Ice for 2 min.
9. Add 400 ul room temperature S.O.C. broth.
10. Agitate cultures gently, 37oC, 1 h. Tube turners or rockers are good for this.
11. Spread all 500 ul on a LB + Ampicillin plate for the transformations from ligations. Spread only 50 ul for the transformation of circular plasmid.
12. Incubate 37oC O/N. Invert plates if all the liquid goes into the plate. Incubate wet plates right side up.

Thursday 11/21 (any time between 2 to 3 p.m.?)

1. Count colonies on the ligation plates. Compare to the number of colonies on vector only control plate. If you have twice as many colonies in the ligation transformation plate, then 50% should contain the insert!
2. Make 4 - 10 ml overnight cultures of LB + Amp in 50 ml tubes. Label tubes 1-4 (initials and date).

3. Inoculate @ with 1 colony: from vector only plate (1 culture) or from ligation plate (3 cultures).

4. Incubate with strong agitation (200 rpm) at 37°C O/N.

Lab 12 11/22

Plasmid DNA Miniprep

NOTE 1: Phenol for DNA is buffered with Tris to pH8 for optimal partitioning. For RNA, Phenol is water-saturated and is ~pH5. Use the correct phenol for your nucleic acid!!!

NOTE 2: ALSO, Phenol is corrosive - causes burns! Be careful! Wear safety glasses!!!

NOTE 3: CHCl₃ (Chloroform) dissolves things like styrofoam and polystyrene - use glass graduated pipets and polypropylene 15 & 50 ml tubes.

In a short, mind-numbing period of tube shuffling, one can extract plasmid DNA for analysis of desirable clones

1. Spin down 1.5 ml overnight culture cells in a 1.5 ml tube - (DISCARD all materials from steps 1 -3 that are contaminated with cells in BIOHAZARD BAGS.)

2. Save the rest of the overnight culture at 4°C - IF it is a good clone, you'll want to make a glycerol stock for long term storage and a streak plate for short term use.

3. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.

4. Resuspend the pellet by trituration (pipetting up and down) in 150 µl of an ice-cold solution of:

50 mM glucose

10 mM EDTA

25 mM Tris/ HCl (pH 8.0)

Add 4 mg/ml lysozyme (added freshly to the solution)

5. Store for 5 minutes at room temperature. The top of the tube need not be closed during this period.

6. Add 300 µl of a room temperature solution of: 0.2 N NaOH + 1% SDS

Close the top of the tube and mix the contents by inverting the tube rapidly two or three times. Do not vortex. Store the tube on ice for 5 minutes.

7. Add 225 µl of an ice-cold solution of potassium acetate (~pH 4.8). [This reagent was made

up as follows: To 60 ml of 5 M potassium acetate, add 11.5 ml of glacial acetic acid and 28.5 ml of H₂O. The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.]

Close the cap of the tube and vortex hard, put on ice, vortex again. Store on ice for 5 minutes.

8. Centrifuge for 15 minutes in a micro-centrifuge at 20°C.

9. Transfer 600 µl supernatant to a fresh tube. (Avoid all white, solid garbage).

10. Add an equal volume of phenol/chloroform pH8. Mix by vortexing. After centrifuging for 2 minutes in an Eppendorf centrifuge, transfer the aqueous phase to a fresh tube.

11. Add two volumes of ethanol at room temperature. Mix by vortexing. Stand at room temperature for 2 minutes.

12. Centrifuge for 5 minutes in an Eppendorf centrifuge at room temperature.

13. Remove the supernatant. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away.

14. Add 1 ml of 70% ethanol. Vortex briefly and then centrifuge.

15. Again remove all of the supernatant. Air dry the pellet briefly (5 minutes) after wiping away residual ethanol with a Kimwipe.

16. Add 50 µl of TE (pH 8.0). Vortex and incubate at 37°C to solubilize DNA (about 5 minutes).

17. Store at -20°C until next lab session.

Lab 13 12/6

Miniprep plasmid DNA Restriction and Gel Analysis

A. To analyze the four plasmid preps from overnight cultures, we'll restrict each with EcoRI and Bam HI to see if the 400bp insert is present.

1. We will cut inserts out of the vectors for each plasmid prep to verify it. Best pipetting technique minimizes pipetting steps (and, thus, work and error). So, to set up a set of 20 µl restriction digestions, make a master mix of common components:

In single reaction x5 = in master mix

6 µl H₂O x5 = 30 µl

2 µl 10X buffer x5 = 10 µl

1ul 1mg/ml RNase x5 = 5 ul 1mg/ml RNase

0.5 ul Bam H1 (10 U/ul) x5 = 2.5 ul Bam H1

0.5 ul EcoR1 (10U/ul) x5 = 2.5 ul EcoR1

Use 10ul/rxn

Mix gently. Pipet 10ul into 4 tubes.

Add 10 ul of individual plasmid preps, one to each tube. Incubate 1 h at 37°C. ALSO Prepare one uncut plasmid sample: 10 ul plasmid + 10 ul TE and incubate at 37°C. After incubations, add 2 ul 10x DNA dye to each of the five tubes. Store leftover plasmid at -20°C.

2. Make a 1% agarose gel + 0.5 ug/ml EtBr in 1X TAE. Use a mid-size apparatus and two 14-well combs.

3. Add 2 ul 10X DNA dye to @ digest.

4. Load gel with uncut plasmid (1 sample) and 4 plasmid digests. Also load Lambda Hind III EcoR1 markers (1 ug) in one well of top and bottom halves of each gel.

5. Run at 120V, 1 h.

6. Photograph under UV light.

7. By comparing the bands in plasmid lanes with Lambda standards, one can describe

a. The molecular size of the fragment

b. The [DNA] of each fragment, and

c. the molecular form (circular vs. linear) of the fragments

For (a), compare migration distance to that of Lambda standard fragments - estimate bp size.

For (b), compare brightness of bands to those of Lambda fragments - estimate ng. Divide by 10 ul (amount of sample loaded) to get [DNA]. This is often more reliable quantitation than A260 measures!!!

For (c), note that uncut plasmids run fast as two forms, supercoiled and nicked circular. The restricted plasmid from vector only transformation shows that linear DNA is slower. Since markers are linear DNAs, their migration only relates to other linear DNAs.

NOTE: MARK DYE POSITIONS ON GEL PHOTOGRAPHS. Label all photos completely!
Name, date, identity!!!

Lab Clean-up:

Biohazard waste

Discard phenol in appropriate bottles

Samples - save?

Return: 10 ml pipettors

APPENDIX

Use of Micropipettors

1. Choose the correct pipet. For volumes:

1-20 μ l P20

20-200 μ l P200

200 - 1000 μ l P1000

2. Set the desired volume by holding the pipetman in one hand and turning the volume adjustment knob until the correct volume shows on the indicator. For best precision, always approach the desired volume by dialing downward (at least one-third revolution) from a larger volume setting.

3. Attach a new tip to the shaft of the pipet. Press tip on firmly to ensure airtight seal. Choose the correct tip.

P20 yellow tip

P200 yellow tip

P1000 blue tip

4. Depress plunger to first positive stop. Hold pipetman vertically and immerse disposable tip into sample liquid 2mm.

5. Allow the push button to return slowly to the up position. Never permit it to snap up.

6. Wait 1 or 2 seconds to ensure that the full volume of the sample is drawn into the tip.

7. Withdraw tip from the sample liquid. Wipe the sides of the tip on the sides of tube to remove any remaining liquid.
8. To dispense the sample, place the tip end against the side wall of the receiving vessel and depress the plunger slowly to the first stop. Then depress the plunger to the second stop to expel any residual liquid in the tip.
9. With the plunger fully depressed, withdraw pipetman from the vessel. Then allow the plunger to return to the top position.
10. Discard tip by depressing the tip ejector button. A fresh tip should be used for each sample.

Terribly Difficult Calculations

1. Molar solutions

1 M (mole per liter) means the solution has 1 molecular weight mass (g) per volume (liter) of soln.

A mole is a number of molecules:

6.022×10^{23} , Avogadro's number

To make 500 mls of 0.5 M NaCl (NaCl is 58.55 g/mole) you need (0.5 liters)

(0.5 mole) = 0.25 mole

liter)

$0.25 \text{ mole} \times 58.55 \text{ g/mole} = 14.6 \text{ g}$

So: Add 14.6 g NaCl power and bring final volume to 500 ml with H₂O.

2. We typically work with concentrated stock solutions. For example, our Tris/acetate/EDTA (TAE) is made as a 50X stock. We run gels in 1000 mls of 1X TAE. The way I do DILUTION PROBLEMS is:

$[\text{Stock}] \times y = [\text{Desired}] \times \text{Desired volume}$; where y is the volume of stock. To find Y needed to make 1 li of 1X TAE from a 50X stock:

$$50X \times y = 1X \times 1000 \text{ ml}$$

$$y = 1X/50X \times 1000 \text{ ml} = 20 \text{ ml}$$

So add 20 ml 50X TAE to a 1 liter graduated cylinder. Bring volume to 1 li w/ dH₂O.

3. Note: Dilutions are applicable to problems of pipetting very small amounts. If you want to add 0.2 ul, dilute the material 1:9 and pipet 2 ul with a P-20.

4. Percentage solutions should have a (v/v) or (w/v) or (w/w) following.

a. (v/v) relates volume to volume, indicating both components are liquids: e.g. 100 mls of 75% (v/v) EtOH is made with 75 mls EtOH + 25 ml H₂O

b. (w/v) indicates solid to liquid ratio: e.g., 10 mls of 10% (w/v) ammonium persulfate (APS) is made w/ 1 g of APS to 10 ml final volume with water.

c. (w/w) is rare, indicating a weight to weight relationship. To make 10 mls of a 10% (w/w) APS soln, you could weigh 1 g APS on a scale and then add water until solution weight is 10 g. (That would be 9 g = 9 mls since density of H₂O is 1 g/ml).

5. Of course, these calculations can be combined. For example, to make 500 mls of 0.5 M NaCl in 1X TAE,

Combine 14.6 g NaCl with 10 mls 50X TAE. Bring volume to 500 mls with H₂O.

Easy!

PROBE TEST GEL

(short, fat sequencing gel)

5% acrylamide/urea gel

25.5g urea

19.5ml H₂O

12ml 5X TBE

7.5ml 40% (w/v) acrylamide (19:1 acryl:bis)

60ml final vol.

Heat to 37°C to dissolve urea

Cool to below RT

Filter (~optional)

Add 400 ul 10% APS (less than 1 week old, make 1ml)

50 ul TEMED

Clean gel plates with soap, rinse with H₂O extensively, then wipe with EtOH and Kimwipes. Set up plates as in Fig. 2 (except don't clamp over sponge).

Pour into 1.5mm thick vertical gel slab. Add comb.

Bubbles = Bad

Should polymerize in 15 min.

Rinse wells with 1X TBE immediately after pulling the comb and just prior to loading.

Samples in 80% (v/v) formamide loading dye

Heat 68°C 5 min for RNA. For DNA, 94°C 5 min.

Run at 25 to 35 mamps.

LIGATION IN LOW-MELT AGAROSE

1. Run restricted vector and insert DNAs on 0.8% low-melt agarose gel in TAE buffer. (If vector is cut with a single enzyme, treat with calf intestinal phosphatase (CIP) prior to gel run to prevent recircularization of the vector). Cut out bands and remove excess agarose while visualizing EtBr-staining under long wave UV light.

2. Melt DNA + agarose 70°C, 10 min.

3. For @ ligation reaction, combine H₂O and DNA + agarose in 9 ul volume (usually 1 ul vector and 3 to 8 ul insert). Put at 37°C for 2-3 min. Perform a vector ligation control reaction without insert.

4. Add 11 ul ice-cold ligase mix composed of

2 ul 10X Ligase Buffer

1 ul Ligase

7 ul H₂O

1 ul 10mm ATP

for each reaction. Flick tube immediately! Immediately slam on ice. Reactions will gel while ligation occurs.

5. Incubate 15oC, O/N.

6. Melt ligation 70oC, 10 min. Transform with 1 ul.

Dye Migration Related to bases in a denaturing gel (d) or base pairs in a non-denaturing gel (n):

Acrylamide % Bromphenol Blue Xylene Cyanol

3.5% n 100 -

5% n 65 -

5% d 35 130

6% d 26 106

8% n 45 -

8% d 19 75

10% d 12 55

12% n 20 -

20% n 12 -

e.g., in a 5% acylamide + urea gel (denaturing), Bromphenol Blue comigrates with 35 base nucleic acids.

Agarose Gels for DNA

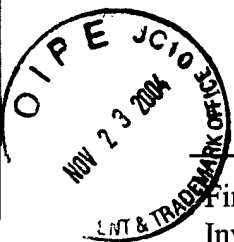
1% 1.5%

1g Agarose 1.5g Agarose

100ml 1X TAE 100ml 1X TAE

10ul 10mg/ml EtBr 10 ul 10mg/ml EtBr

Mix in 250ml beaker, cover with Saran Wrap, heat in microwave until solution boils 3 times.
Allow to cool to 60°C, pour gel.



First Named

Inventor : Michael E. Spurlock

Appln. No. : 09/932,888

Filed : August 20, 2001

Title : Porcine Leptin Protein, Antisense and Antibody

Docket No. : LL31.12-0016

Group Art Unit: 1647

Examiner: C. J. Saoud

EXHIBIT F

of

DECLARATION

submitted under 37 C.F.R. 1.132

Hyperdictionary @ [www.hyperdictionary.com/dictionary/Denhardt's + solution](http://www.hyperdictionary.com/dictionary/Denhardt's%20solution);
obtained from the Internet on November 12, 2004



English Dictionary Computer Dictionary Thesaurus Dream Dictionary Medical Dictionary

Search Dictionary:

Meaning of DENHARDT'S SOLUTION

Biology Dictionary

Definition: A solution commonly used during probe hybridizations that involve filters (such as Southern, Northern, or Western blots). The solution contains ficoll, bovine serum albumin, polyvinylpyrrolidone (PVP), and a high concentration of nonspecific DNA so the probe won't hybridize nonspecifically.

Websites:

- **Find the Best Sites For Denhardt's Solution With Starware**

Starware search is an excellent resource for quality sites on Denhardt's Solution and much more! Starware also provides related listings for Denhardt's Solution.
search.starware.com



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EXHIBIT G

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Information Page Entitled "Sheet for Eppendorf® Sheared Salmon Sperm DNA"
located at <http://www.brinkmann.com/product.asp?path=115&ref=136>;
obtained from the Internet on November 12, 2004

SEARCH :

Eppendorf® Sheared Salmon Sperm DNA

All Products → Molecular Technologies → Molecular Biology Reagents → Molecular Biology Reagents

Description

Group

■ Print

■ Email This Page

■ Manual

■ FAQ

**Storage at -20 °C**

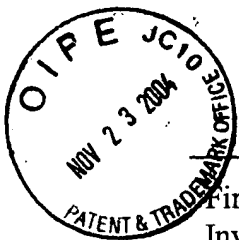
Applications

- Hybridization
- Nucleic acid precipitation

Sheared Salmon Sperm DNA is used as a blocking agent to reduce the background in hybridization experiments.¹ It may be used as a carrier during DNA and RNA precipitation with alcohol. The Sheared Salmon Sperm DNA is supplied at 10 mg/ml in sterile DNase- and RNase-free Molecular Biology Reagent.

The Salmon Sperm DNA is sheared by sonication, but it is not fully denatured as supplied. Sheared Salmon Sperm DNA should be heated to 100 °C for 5 minutes and then quickly cooling it in an ice bath before use is recommended. The Sheared Salmon Sperm DNA should be used at a concentration of 1 µg/ml in hybridization solutions. The DNA is approx. 200–2,500 bp in length as determined by 1% agarose gel electrophoresis.

¹ Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. "Molecular Cloning: A Laboratory Manual", 2nd edition. Cold Spring Harbor, NY: pp 9.8–9.49.



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EXHIBIT H

of

DECLARATION

submitted under 37 C.F.R. 1.132

Instructions posted at <http://www.clarkson.edu/class/by412/word/Northern%20hybridization.doc>
(obtained from the Internet on November 12, 2004) for Molecular Biology Lab #17
(Northern Transfer and Hybridization Experiment #2: Labeling Probe and Hybridization)
taught in Fall, 2004 at Clarkson University in Potsdam, New York by Craig Woodworth

Molecular Biology Lab 17

Northern Transfer and Hybridization Experiment #2: Labeling Probe and Hybridization

Background:

The process of labeling and hybridization of Northern blots is performed in a series of steps over several days. The first step is called prehybridization. During this step the membrane containing the RNA is pretreated with a buffer containing blocking reagents such as albumin or salmon sperm DNA. These treatments block any nonspecific 'hot spots' on the membrane that might bind to probe nonspecifically. Often, prehybridization is performed for 30 min using the same buffer that will be used subsequently for hybridization. If prehybridization is inadequate, the blot may have high background with nonspecific probe binding.

Probe labeling is the next step. There are several methods for labeling probes, but all methods require that the DNA molecule first be denatured to separate the 2 complementary strands. This is usually performed by boiling the probe DNA for 5 min then rapidly cooling. Rapid cooling helps to prevent renaturation of the complementary strands.

The most sensitive method of detection uses probes that are labeled with radioactive ^{32}P by random priming or nick translation. These methods add a ^{32}P labeled nucleotide (often deoxy CTP) throughout the probe DNA molecule. The labeling reaction is then passed through a column that binds unincorporated nucleotides and allows the radioactive DNA to elute. This probe is very hot (1×10^8 cpm/ μg DNA) and can be used to detect single copy genes with ease. The radioactive DNA that binds specifically to the probe is detected by placing the membrane next to X-ray film, or analyzing the membrane in a phosphorimager. The use of ^{32}P is also associated with potential hazards of external contamination. ^{32}P is very high-energy beta emitter and the researcher must take precautions to shield the body from radiation. Usually this is done by working behind a small Plexiglas shield. All of the experimental waste material must be carefully retained and then disposed of and careful records must be kept. All sources of ^{32}P must be kept under lock and key in the lab (i.e. lab doors are always locked when using ^{32}P) which can be an inconvenience. ^{32}P is rather expensive and has a short half-life. In fact, probes must be used within several days or they decay so much that they are no longer useful.

Another method for labeling probes is by utilizing chemiluminescent detection methods. These are slightly less sensitive than ^{32}P and are not widely used for Southern or Northern blotting when sensitivity is important. However, there is little or no hazard associated with chemiluminescent probes and they have achieved wide use in Western blot applications. This method works by directly labeling the probe DNA with an alkaline phosphatase enzyme. This is achieved by first denaturing the probe DNA and then adding the enzyme along with a cross linking reagent. The alkaline phosphatase labeled DNA that specifically binds to the RNA on the blot is detected by placing the washed blot in a special substrate solution that alkaline phosphatase can dephosphorylate. This reaction is

associated with the release of chemiluminescence which can be detected with X-ray film or a phosphoimager. On the positive side, there is no biological hazard. You can leave the lab doors open, drink coke in the lab, and throw the waste in the regular trash. The probes also have a long half-life and they can be stored for weeks to months.

A third method of detection is colorimetric. This method is very similar to the chemiluminescent protocol, except that the sensitivity is much lower. The probe DNA is labeled with an alkaline phosphatase enzyme by cross-linking and the labeled DNA hybridizes specifically to the complementary RNA on your membrane. The color detection method uses a substrate for the alkaline phosphatase enzyme that becomes insoluble and turns blue when it is cleaved. This method is inherently less sensitive than chemiluminescence or radioactivity. However, it has the advantage that it requires no darkroom or film development reagents.

The third step is hybridization of the labeled probe to the membrane. This is usually performed in a hybridization oven, which carefully regulates temperature and allows the blot to turn constantly so that it is continually bathed in new hybridization solution. There is definitely an optimal temperature for performing hybridization reactions. If the temperature is too low (low stringency) the DNA and RNA strands can join rather easily and they often are able to join even if the complimentary strands don't match completely. For example, at low stringency, a probe for the beta actin gene might cross hybridize with an RNA for alpha actin. The alpha actin RNA from humans might hybridize to the alpha actin probe from a dog. The match doesn't need to be perfect. This can be an advantage if one is searching for RNAs from a new gene family in the same species (maybe you want to use low or relaxed stringency to look for new actin genes). It is also useful if you are searching for an RNA from another species. On the other hand, you can raise the temperature too high (high stringency) so that it is very difficult for any RNA and DNA to hybridize. This is because the temperature is too close to the melting temperature so the hybrids that form are easily denatured again. Under these circumstances, only those sequences that are perfectly matched can form stable hybrids.

How do you choose the right temperature? It depends on many factors including the GC content of the DNA (GC rich DNA melts differently than AT rich DNA), salt concentration (high salt means lower stringency), or the presence of formamide (this lowers the melting temp of DNA). You can also control for stringency of hybridization at the next step, which is washing the unbound probe away from the blot. If you wash at high temperature or low salt concentration, you will remove any hybrids that are not perfectly matched. If you wash at low temperature and/or in high salt, you are leaving many imperfect hybrids on your membrane. Usually, washing proceeds with a low stringency wash at first to remove most of the unbound probe. This is followed by a higher stringency wash. One advantage of using radioactive probes, is that you can easily monitor how 'hot' or radioactive your blot is by simply checking it with the Geiger counter. If it were too 'hot' you would use a more stringent wash. With chemiluminescent or colorimetric detection, you will not know whether you have too much background until you actually develop the blot.

Objectives:

The objective of this lab is to provide experience in labeling DNA with a chemiluminescent probe and using the probe in a Northern hybridization reaction.

Materials:

AlkPhos Direct Labeling and Detection Kit

Hybridization buffer

Primary wash buffer (2M urea, 0.1% SDS, 50 mM sodium phosphate at pH 7, 150 mM Sodium chloride, 1 mM magnesium chloride, 0.2 % blocking reagent)

10X Secondary wash buffer (1M Tris pH 10, 2M sodium chloride)

Alkaline Phosphate Conjugate Substrate Kit

Hybridization oven set at 55°C

Hybridization bottles and nylon mesh

37°C water bath

55°C water bath

Shaker platform

Plastic dishes for washing blots

Pipettors and yellow tips

Ice water bath

Eppendorf microcentrifuge

Previously prepared blot with RNA sample

Eppendorf tubes

Boiling water bath

Dark room with developer and fixer solutions

Film cassettes

X-ray film

Plastic wrap

Methods:**Prehybridization:**

1. Preheat the required volume of hybridization buffer to 55°C in the hybridization oven. Heat enough buffer for 0.25 ml/cm² of membrane. Also, preheat the glass hybridization bottle containing 15 ml of deionized water.
2. Rehydrate the nylon membrane in water for 5-10 min.
3. Place the blot on a sheet of nylon mesh that is slightly larger (1-2 mm on each side). Make sure that the RNA side of the blot is facing up (check under a UV light if you are not sure). Carefully, roll the blot and mesh and slip the roll into the glass hybridization bottle. The RNA side should face into the hybridization chamber.
4. Place the cap on the tube and hold the tube horizontally. Turn the tube slowly until the membrane unrolls inside the tube and is applied to the walls of the tube.

Inspect the tube and membrane carefully to make sure that there are no air bubbles between the tube wall and the membrane. If air bubbles are present, pull the filter out and start again. Any air bubbles will lead to excess background.

5. Once the filter is applied to the wall of the tube, pour out the water and add about 15 ml of hybridization buffer. Cap the tube and place it into the clips in the hybridization oven. Turn on the speed control such that the bottle turns slowly through the oven. Check to see that the bottle is attached evenly and that the hybridization fluid covers the bottom of the bottle.
6. Allow the blots to prehybridize (before adding the probe) for approximately 30 min. This step is important to block any nonspecific reactive sites on the membrane. Lack of adequate prehybridization can lead to high background due to nonspecific binding of probe to the membrane.

Preparation of probe:

7. Prepare the labeled hybridization probe. Dilute 20 μ l of cross linker solution with 80 μ l of the water supplied with the kit. This working concentration should be kept on ice.
8. Dilute HPV-16 DNA to a concentration of 10 ng/ μ l using the water supplied with the kit.
9. Place 10 μ l of the diluted DNA sample into an eppendorf tube and denature the DNA by heating for 5 min in a boiling water bath.
10. Immediately cool the DNA on ice for 5 min. Briefly spin the sample in a microcentrifuge to collect the contents at the bottom of the tube.
11. Add 10 μ l of reaction buffer to the cooled DNA and mix thoroughly but gently. Be sure to keep the tube on ice.
12. Add 2 μ l of labeling reagent and mix thoroughly but gently.
13. Add 10 μ l of the cross linker working solution. Mix briefly and spin to collect the contents at the bottom of the tube.
14. Incubate the reaction at 37°C for 30 min. The probe can be used immediately or kept on ice for up to 2 hours.

Hybridization reaction:

15. Add labeled probe to the buffer used for prehybridization. Use about 5-10 ng of probe per ml of hybridization buffer. Avoid placing the probe directly on the blot.

Remove a small amount of hybridization fluid from the bottle and mix with the probe before returning this mixture to the bulk of the hybridization buffer.

16. Hybridize at 55°C for 2 days in the hybridization oven.

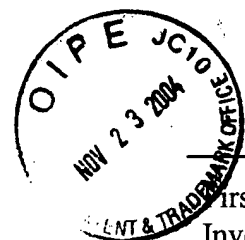
Post hybridization washes:

17. Preheat the primary wash buffer to 55°C (do not overheat!). Use this in excess at a volume of 2-5 ml per cm² of membrane.
18. Carefully remove the roller bottle from the oven. This is easier if you briefly switch off the motor for rotation, and then turn it on again after the bottle is removed. Pour out the hybridization buffer from the roller bottle and add the preheated primary wash buffer (fill the tube halfway).
19. Place the tube with wash buffer back in the hybridization oven and wash for 10 min at 55°C.
20. After 10 min, remove the roller bottle and pour out contents. Add more prewarmed primary hybridization buffer and allow washing in the oven for another 10 min at 55°C.
21. Carefully remove the membrane and mesh from the hybridization bottle using tweezers. Place the membrane in a plastic wash dish with 100-200 ml of secondary wash buffer. Wash with gentle agitation on a shaker platform for 10 min at room temperature. Several blots may be washed in the same secondary wash buffer provided that there is enough volume to allow them to move freely.
22. Pour off the wash buffer and add 100-200 ml of fresh secondary wash buffer. Shake gently for an additional 10 min at room temperature.

Chemiluminescent signal detection:

23. Allow the membrane to drain and briefly dab any excess fluid away with a paper towel
24. Add 3ml of chemiluminescent substrate to your blot and allow it to saturate the membrane. This can be done by placing the blot and substrate in an empty yellow tip box and rocking back and forth.
25. Dab excess substrate from blot using a paper towel and wrap the membrane carefully in plastic wrap. Tape the wrapped membrane to the inside of an X-ray film cassette.
26. Bring the cassette and an unopened X-ray film into the darkroom. Turn on the safelight and shut the door. Check to see that no light is coming in from outside.

27. Open the X-ray film and carefully place a sheet into the cassette so that it covers the blot. Close the cassette cover and allow the film to be exposed for 1 hour. Make sure that the cassette snaps closed (listen for the click).
28. After exposure, return to the darkroom and close the door. Wear latex gloves. Turn on the safelight and open the cassette. Place the film in the developer for approximately 30 sec with periodic agitation. You should see the image of the blot appear.
29. Transfer the developed film to water to remove excess developer and agitate for 2 to 3 min.
30. Place the film in fixer and leave for 2 to 3 min. After this, you can turn on the light. After 30 min, you can place the film in water and wash for 1 to 2 hours. Most modern molecular biology labs have an automated film processor that automatically develops films in 1 to 2 min.



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(54) Title: SYSTEMS AND METHODS FOR IMPROVING PROTEIN AND MILK PRODUCTION OF DAIRY HERDS

(57) Abstract: The present invention provides for a direct correlation between milk production in livestock animals and the presence of alleles of a gene encoding an adipocyte-specific polypeptide, termed leptin, which gene is hereinafter referred to as *ob*. The invention also provides novel compositions consisting essentially of specific oligonucleotides that are useful as primers to amplify particular regions of the genome during enzymatic nucleic acid amplification, thus providing a rapid, sensitive and specific method for the detection of the *ob*-gene polymorphism which may be present in a specimen. The invention further provides for methods of screening bovine to determine those having predictably more milk productivity and advantageously selecting those livestock for future breeding and management purposes based on the *ob* polymorphisms.

WO 2004/083456 A1

**SYSTEMS AND METHODS FOR IMPROVING PROTEIN AND MILK
PRODUCTION OF DAIRY HERDS**

INCORPORATION BY REFERENCE

5 This application is a continuation-in-part of copending application USSN 10/770,307,
filed February 2, 2004, which claims priority to U.S. Provisional Application Serial No.
60/466,523 entitled "METHOD FOR IMPROVING EFFICIENCIES IN LIVESTOCK
PRODUCTION", filed April 29, 2003, and U.S. Provisional Application Serial No.
60/509,775 entitled "METHOD FOR IMPROVING FEED CONVERSION EFFICIENCY
10 IN LIVESTOCK PRODUCTION", filed October 8, 2003. This application also claims
priority to Canadian Patent Application No. 2,422,437 entitled : "IMPROVING PROTEIN
AND MILK PRODUCTION OF DAIRY HERDS", filed March 18, 2003 and to U.S.
Provisional Application Serial No. 60/456,489 entitled : "PROTEIN AND MILK
PRODUCTION OF DAIRY HERDS", filed March 21, 2003. The foregoing applications,
15 and all documents cited therein or during their prosecution ("appln cited documents") and all
documents cited or referenced in the appln cited documents, and all documents cited or
referenced herein ("herein cited documents"), and all documents cited or referenced in herein
cited documents, together with any manufacturer's instructions, descriptions, product
specifications, and product sheets for any products mentioned herein or in any document
20 incorporated by reference herein, are hereby incorporated herein by reference, and may be
employed in the practice of the invention.

FIELD OF THE INVENTION

 The present invention relates to a method of managing livestock animals by selecting
the animals according to a specific genotype and, in particular, to a method for selecting
25 animals for inclusion in a group of animals according to variations in the *ob* gene so as to
select animals with a greater propensity for milk production. The present invention relates to
a method of identifying animals of a first genotype that produce more milk and milk protein
as compared to the animals of a second different genotype. By selecting animals of the first
genotype for inclusion in a group of animals, thereby increasing the number of such animals
30 in the group compared to a conventionally selected group, the amount of milk and milk
protein can be increased.

 Also provided by the present invention are methods of using genetic markers relating
to the regulation of energy intake and metabolism in growing, finishing, lactating or

nonlactating, and gestating livestock, methods for identifying such markers, and methods of screening livestock to determine those having predictably more uniform fat deposition and altered milk production and milk components, as well as advantageously selecting those livestock for future breeding and management purposes based on polymorphisms. The markers are based upon the presence or absence of certain polymorphisms in the *ob* gene. Also disclosed herein are oligonucleotides that can be used as primers to amplify specific nucleic acid sequences of the *ob* gene. The present invention also provides oligonucleotides that can be used as probes in the detection of amplified specific nucleic acid sequences of the *ob* gene.

10 **BACKGROUND OF THE INVENTION**

Leptin, a 16-kDa adipocyte-specific polypeptide is expressed predominantly in fat tissues of those animals in which it has been detected, which animals include livestock species such as cattle, pigs, and sheep. Leptin is encoded by the *ob* (obese) gene and appears to be involved in the regulation of appetite, basal metabolism and fat deposition. Increased plasma concentrations of leptin in mice, cattle, pigs and sheep have been associated with decreased body fat deposition and appetite, and increased basal metabolism levels (Blache *et al.*, J Endocrinol. 2000 Jun;165(3):625-37; Delavaud *et al.*, J Endocrinol. 2000 May;165(2):519-26 and Ehrhardt *et al.*, J Endocrinol. 2000 Sep;166(3):519-28). Similar phenotypic characteristics have also been found to be associated with leptin mRNA levels in adipose tissue (Ramsay *et al.*, J Anim Sci. 1998 Feb;76(2):484-90 and Robert *et al.*, Can. J. Anim. Sci. 1998;78:473-82). Consistent with those observations, it has been shown that administration of exogenous leptin dramatically reduces feed intake and body mass of mice, chickens, pigs and sheep (Barb *et al.*, Domest Anim Endocrinol. 1998 Jan;15(1):77-86; Halaas *et al.*, Science. 1995 Jul 28;269(5223):543-6; Henry *et al.*, Endocrinology. 1999 Mar;140(3):1175-82 and Raver *et al.*, Protein Expr Purif. 1998 Dec;14(3):403-8).

The *ob* gene that has been mapped to chromosome 6 in mice (Friedman & Leibel, Cell. 1992 Apr 17;69(2):217-20), chromosome 7q31.3 in humans (Isse *et al.*, J Biol Chem. 1995 Nov 17;270(46):27728-33) chromosome 4 in cattle (Stone *et al.* Mamm. Genome 1996;7: 399-400), and chromosome 18 in swine (Neuenschwander *et al.*, Anim Genet. 1996 Aug;27(4):275-8 and Saskai *et al.*, Mamm. Genome 1996;7:471). Sequences have been determined for the said gene from mice (Zhang *et al.*, Nature. 1994 Dec 1;372(6505):425-32), cattle (U.S. Patent No. 6,297,027), pigs (U.S. Patent No. 6,277,592 and Neuenschwander

et al., Anim Genet. 1996 Aug;27(4):275-8), and humans (U.S. Patent No. 6,309,857) and there is significant conservation among the sequences of *ob* DNAs and leptin polypeptides from those species (Bidwell *et al.*, Anim. Biotech. 1997;8:191-206 and Ramsay *et al.*, J Anim Sci. 1998 Feb;76(2):484-90).

5 It has been demonstrated that plasma leptin concentrations are significantly diminished in animals homozygous for mutant alleles of the *ob* gene (*ob⁻/ob⁻* animals), which alleles do not encode functional leptin, compared to wild-type (*ob⁺/ob⁺*) controls. Mutations in the coding sequences of the *ob* gene causing alterations in the amino acid sequence of the leptin polypeptide, have been associated with hyperphagia, hypometabolic activity, and
10 excessive fat deposition; *i.e.*, a phenotype characterized by larger body size; a fat phenotype (Zhang *et al.*, Nature. 1994 Dec 1;372(6505):425-32).

Fitzsimmons *et al.* (Mamm Genome. 1998 Jun;9(6):432-4) reported evidence of three alleles of a microsatellite marker located proximal to the *ob* gene in cattle that occurred with significant frequency in bulls of several breeds (Angus, Charolais, Hereford and Simmental)
15 and comprising 138, 147 and 149 base pairs (bp). The 138-bp and 147-bp alleles, respectively, occurred most frequently. Further, it was determined that occurrence of the 138-bp allele was positively associated with certain carcass characteristics; increased average fat deposition, increased mean fat deposition, increased percent rib fat, and decreased percent rib lean. Thus, bulls homozygous for the 138-bp allele exhibited greater average fat
20 deposition than heterozygous animals and such heterozygotes exhibited greater average fat deposition than bulls homozygous for the 147-bp allele.

Subsequently, Buchanan *et al.* (Genet Sel Evol. 2002 Jan-Feb;34(1):105-16) identified a cytosine (C) to thymine (T) transition within an exon (exon 2) of the *ob* gene, corresponding to an arginine (ARG) to cysteine (CYS) substitution in the leptin polypeptide.
25 The presence of the T-containing allele in bulls was associated with fatter carcasses than those from bulls with the C-containing allele.

Single nucleotide polymorphisms have also been detected in the porcine *ob* gene and certain of those polymorphisms have been found to be associated with feed intake and carcass traits (Kennes *et al.*, Anim Genet. 2001 Aug;32(4):215-8 and Kulig *et al.*, Arch. Tierz. Dummorscorf 2001;44:291-296). Means of selective amplification of bovine gene are in
30 U.S. Patent No. 6,297,027.

It is possible to distinguish *ob* genotypes by cloning and sequencing DNA fragments from individual animals, or by other methods known in the art. For example, it is possible to distinguish *ob* genotypes by employing synthetic oligonucleotide primed amplification of *ob* gene fragments followed by restriction endonuclease digestion of the amplified product using
5 a restriction enzyme that cuts such product from different *ob* alleles into discrete product fragments of differing length. Such discrete product fragments could then be distinguished using electrophoresis in agarose or acrylamide, for example. The *ob* alleles identified by Buchanan *et al.* (Genet Sel Evol. 2002 Jan-Feb;34(1):105-16) were distinguished by such means using a mismatch PCR-RFLP strategy wherein, the C-containing allele (as above)
10 yields DNA fragments of 75 and 19 bp following digestion of the amplicon with Kpn 2I, and the T-containing allele (as above) is not cut.

In managing livestock animals using present methods, visible characteristics or phenotypic traits are used to predict how an animal will grow, and thus how the animal should be fed to most profitably achieve market condition. The object of a livestock industry
15 is to convert feed into meat, and much is known about growth patterns of livestock.

Body condition is a determinant of market readiness in commercial livestock feeding and finishing operations. The term body condition is used in livestock industry in reference to the state of development of a livestock animal that is a function of frame type or size, and the amount of intramuscular fat and back fat exhibited by an animal. It is typically
20 determined subjectively and through experienced visual appraisal of live animals. The fat deposition, or the amount of intramuscular fat and back fat on an animal carcass, is important to industry participants because carcasses exhibiting desired amounts and proportions of such fats can often be sold for higher prices than carcasses that exhibit divergences from such desired amounts and proportions.

25 Furthermore, the desired carcass fat deposition often varies among different markets and buyers, and also often varies with time in single markets and among particular buyers in response to public demand trends with respect to desired of fat and marbling in meat.

Weight gain by a livestock animal during its growth and development typically follows a tri-phasic pattern that is carefully managed by commercial producers, and finishers.
30 The efficiency of dietary caloric (feed) conversion to weight gain during an increment of time varies during three growth phases; a first phase of growth comprises that portion of a

livestock animals life from birth to weaning, and is not paid much heed by commercial feeding and finishing operators.

5 A second growth phase comprises that portion of a livestock animal's life from weaning to attainment of musculo-skeletal maturity. Feed conversion efficiency is relatively high

during this phase; livestock producers usually restrict caloric intake, which has the effect of causing this phase to be prolonged but also typically results in animals with larger frames, which is the aim of dietary management during this phase. During the second growth phase weight gain is associated with skeletal mass and muscle mass accumulation primarily.

10 During a third growth phase, after an animal has attained musculo-skeletal maturity, the efficiency of feed conversion is reduced, such that it requires more feed to increase an animal's weight. For example with cattle, during the second phase of growth, a typical steer could convert 5 to 6 pounds of feed into one pound of weight gain. Upon entering the third phase, feed conversion efficiency typically decreases, such that 7 up to 10 or more pounds of
15 feed are required to produce one pound of gain.

During the third phase livestock feeders significantly increase the caloric content of animals' rations. During the third growth phase weight gain is associated with fat accumulation primarily. Again using cattle as an example, with a steer weighing 900 pounds at the end of the second phase, of that 900 pounds, typically 350 pounds will be red meat. At
20 the end of the third phase, the steer would typically weigh 1400 pounds and typically 430 pounds will be red meat.

Keeping the cattle industry as an example, initially a cow/calf operator will breed bulls to cows, birth calves from the cows, and allow the calves to feed on their mother's milk until they are weaned some months after birth. This is the first phase of growth of the calf.

25 After weaning, the calf enters the second stage of growth where it is fed to grow to its full skeletal size. This commonly called the "backgrounding" phase during which musculo-skeletal maturity is achieved. When the animal has reached its full size, it enters the third phase of growth where the fully grown animal puts on weight.

Typically it is at the start of the third stage of growth that the animal enters a finishing
30 feed lot. In the feed lot the object is to feed the animal the proper ration so that it will most quickly obtain the proper market characteristics that are desired at that given time. At present, for instance it is desirable to have beef that is well marbled, i.e., it has considerable

intramuscular fat in the meat. At other times it may be desirable to have lean meat with very little intramuscular fat. The price the feed lot owner attains for his cattle, when he sells to the packer can vary significantly depending on marbling of the meat.

5 Presently, cattle entering a feed lot are divided into groups according to estimated age, frame size, breed, weight and so forth. By doing this the feed lot owner is attempting to group the cattle so that the group can be penned together and fed the same ration and will be ready for market at the same time. Weight and visual clues are the only means possible to sort cattle for feed lot grouping.

10 The phenotype of an animal is defined as the visible characteristics of the animal resulting from the interaction between the animal's genetic makeup and its environment. Thus, present management techniques group cattle according to uniform phenotypic traits and then keep the environment constant for each animal in the group in hopes that the group will together achieve a different phenotype at some later date. Although the genetic makeup of any individual steer is a significant factor in the ability of that individual steer to grow in the
15 same manner as another steer of the same phenotype, this consideration is presently not taken into account by conventional livestock management practices. Instead, cattle are segregated into groups based on phenotypic traits alone even though results of present livestock feeding and grouping methods show the substantial effects that genetic makeup has on the growth of cattle. For example, considerable variation in phenotypes is present at the end of the third
20 phase among cattle that entered the third phase with a substantially uniform phenotype, despite having been subjected to the same environmental factors as with conventional management methods.

It is not uncommon for a pen of cattle, each having a weight within a range of 100 pounds going into a feeding pen, to have weights varying in a range of 300 pounds or more
25 coming out of the pen for slaughter. It is also known that the feed conversion rate of cattle varies to some degree. Since feed represents a major cost to the feed-lot operator, it is more profitable to feed those cattle with a higher feed conversion rate since an animal that converts a ton of feed into 200 pounds of saleable body weight is more profitable than another animal that converts the same ton of feed into only 180 pounds of saleable meat. Presently,
30 however, it is not known how to identify cattle having a higher feed conversion rate, except by measuring feed eaten against weight gained. It is not economically feasible to perform such measurements on each animal entering a commercial feedlot - the numbers of animals

are too great, and individual attention required by the operator to gather the measurements is not possible. In contrast, timing of slaughter is based on the mean visible condition of the group of cattle in each pen, resulting in a wide variation in carcass weight and ensuring that grading premiums for carcasses of a desired condition of weight and fat are not met for a significant number of cattle. In a typical pen, a number of the cattle in the pen would have been at the desired carcass condition earlier, but by the time they are slaughtered they are over fat. Similarly, many cattle could readily achieve the desired carcass condition if fed longer. However, conventional management techniques require that all the cattle in the pen are slaughtered at the same time.

Cattle operators breed bulls to cows, choosing the mating based on signals received through the chain of supply from consumers for those traits that are in demand, for example fat beef or lean beef. European breeds provide carcasses that are typically leaner than British breeds, therefore the cow/calf operator will typically lean to one or the other as demand changes. They also select breeding animals based on visual traits, such as frame size, and anecdotal traits, such as easy calving history. Again, the object is to provide cattle that will command the highest price from the eventual purchaser, such as a backgrounder or feed lot operator.

A dairy cattle operator is faced with similar issues as packers, feeders and cow/calf operators. Dairy cattle are also segregated into groups based upon phenotypic traits even though genotype can affect milk production. In particular, the time period from calving through to peak lactations is the most stressful period in the life of the dairy cow. During this time, the animal usually falls into negative energy balance because the daily feed intake, although increased, is unable to keep pace with the increased energy demand of lactation. Since certain genotypes affect energy balance, management of animals by genotype will be important for efficient dairy production. Furthermore, the animals' genetic predisposition to lay down fat also impacts milk production.

Citation or identification of any document in this application is not an admission that such document is available as prior art to the present invention.

SUMMARY OF THE INVENTION

It is an aspect of the present invention to provide a method for improving efficiencies in milk production.

The invention is based in part on Applicants' finding that the leptin single nucleotide

polymorphism (SNP) associated with increased fat deposition in beef cattle is also associated with lactation performance. The milk and milk protein yield advantage observed in cows homozygous for the T allele, represent an economic advantage to dairy farmers.

5 The present invention also provides a method comprising identifying livestock animals having greater milk productivity by identifying a genetic indicator in the animals that allows management of livestock by genetic selection in addition to phenotype.

The present invention discloses nucleic acid sequences (oligonucleotides) useful as primers and/or probes in the detection of a polymorphism in livestock specimens.

10 The present invention provides oligonucleotide sequences and methods of using them, which permit the prediction of milk production, feed conversion efficiency, and the prediction and modulation of fat deposition in mammals, especially in the bovine species, by looking for mutations in the leptin (*ob*) gene that produces the leptin protein.

Also included in the present invention is a method for detecting the presence of a polymorphism in the nucleic acid molecules for the leptin gene as described herein, or a complementary sequence, in a nucleic acid-containing sample, the method comprising: (a) contacting the sample with an oligonucleotide probe complementary to the sequence of interest under hybridizing conditions; and (b) measuring the hybridization of the probe to the nucleic acid molecule, thereby detecting the presence of the nucleic acid molecule. The above method may additionally comprise before step (a): (c) selectively amplifying the number of copies of the nucleic acid sequence.

20 It is an object of the invention to provide methods of screening livestock to determine those more likely to have increased milk production. The invention also provides for methods of screening livestock to determine those more likely to have predictably uniform fat deposition. Another object of the invention is to provide a method for identifying genetic markers for feed conversion efficiency, a measure of the ability of the animal to convert feed eaten into weight gain, generally measured as the amount of weight gained per pound of feed eaten, or the amount of feed required to put on a pound of weight gain. Yet another object of the invention is to provide a kit for evaluating a sample of livestock DNA for specific genetic markers for increased milk production, and optionally, genetic markers for fat deposition and feed conversion efficiency.

30 Another object of the present invention is to provide oligonucleotides that can be used as primers to amplify specific nucleic acid sequences of the *ob* gene.

It is also an object of the present invention is to provide oligonucleotides that can be used as probes in the detection of amplified specific nucleic acid sequences of the *ob* gene.

Another object of the present invention is to provide oligonucleotides that can be used as primers to amplify DNA sequences from a polymorphism of the *ob* gene. In an advantageous embodiment, the *ob* gene polymorphism is a C to T transition that results in an Arg25 Cys in the leptin protein.

It is the object of the present invention to provide a method for improving efficiencies in livestock production. It is a further objective to provide such a method that comprises grouping livestock animals, such as cattle and pigs, during the period of their retention in a feeding facility according to the genetic predisposition of individual livestock animals to deposit fat, and then feeding the animals in each group substantially uniformly. Optionally, it is yet another object of the invention to decrease the amount of feed needed to produce any given increase in weight of livestock animals in a feedlot by selecting the cattle being fed to increase the occurrence of the T-containing allele of the *ob* gene in the cattle being fed.

It is an embodiment of the present invention to provide such a method comprising determining the genetic predisposition of individual livestock animals to meet particular milk production expectations. In one embodiment, homozygosity or heterozygosity of each animal is determined with respect to alleles, and such animals are segregated into groups based on genotype, *e.g.*, *ob* genotype, and optionally, phenotype. In one embodiment, animals are segregated by phenotype, *e.g.*, frame type and genotype, *e.g.*, homozygosity in respect of a first *ob* allele or homozygosity in respect of a second *ob* allele (*e.g.*, TT or CC animals), or heterozygosity in respect of the first and second *ob* alleles (*e.g.*, CT animals), then feeding and otherwise maintaining animals in a group together and apart from other groups of animals, and ceasing to feed the animals in the group at a time is sustained until the median body fat condition of the animals of that group is of a desired body fat condition.

It is a further embodiment of the present invention to provide such a method of determining homozygosity or heterozygosity of cattle with respect to alleles of the *ob* gene, and sorting the cattle accordingly into three groups, one group homozygous in respect of a first *ob* allele and therefore having the most propensity to lay down fat, a second group homozygous in respect of a second *ob* allele and therefore having the least propensity to lay down fat, and a third group heterozygous in respect of the first and second *ob* alleles and therefore having an intermediate propensity to lay down fat. It is a further object of the

present invention to provide such a method wherein the three groups are further divided according to weight or frame size.

To achieve the objects and in accordance with the purpose of the invention, as embodied and broadly described herein, the present invention provides a method for screening cattle to identify those with a higher propensity towards increased milk production, and also to allow grouping of the cattle to yield a consistent quality grade. A sample of genomic DNA is obtained from the cattle, and the sample is analyzed to determine the presence or absence of a polymorphism in the *ob* gene that is correlated with increased milk production. In an advantageous embodiment, the *ob* gene polymorphism is a C to T transition that results in an Arg25Cys in the leptin protein that is correlated with increased weight gain. In one embodiment, the polymorphism is detected using FRET.

In another embodiment the presence or absence of a specific fragment is assayed for by use of primers and DNA polymerase to amplify a specific region of the gene which contains the polymorphism. In an advantageous embodiment, the *ob* gene polymorphism is a C to T transition that results in an Arg25 Cys in the leptin protein.

In one embodiment, the target nucleic acid is first amplified, such as by PCR, SDA, NASBA, TMA, rolling circle, T7, T3, or SP6, each of which methods are well understood in the art, using at least one amplification primer oligomer. The oligomer may be labeled with a moiety useful for attaching the amplification product to a substrate surface. Following amplification, the amplified dsDNA product may be denatured.

In one aspect, during the hybridization of the nucleic acid target with the anchor probe and/or the sensor probe, stringent conditions may be utilized, advantageously along with other stringency affecting conditions, to aid in the hybridization. In yet another aspect, stringency conditions may be varied during the hybridization complex stability determination so as to more accurately or quickly determine whether a SNP is present in the target sequence. Hybridization stability may be influenced by numerous factors, including thermoregulation, chemical regulation, as well as stringency control, either alone or in combination with the other listed factors.

In one mode, the hybridization complex is labeled and the step of determining amount of hybridization includes detecting the amounts of labeled hybridization complex under stringent and destabilizing conditions. The detection device and method may include, but is not limited to, optical imaging, electronic imaging, imaging with a CCD camera, integrated

optical imaging, and mass spectrometry. Further, the detection, either labeled or unlabeled, is quantified, which may include statistical analysis. The labeled portion of the complex may be the target, the anchor, the sensor or the hybridization complex in toto. Labeling may be by fluorescent labeling selected from the group of, but not limited to, Cy3, Cy5, Bodipy Texas Red, Bodipy Far Red, Lucifer Yellow, Bodipy 630/650-X, Bodipy R6G-X and 5-CR 6G. Labeling may further be accomplished by colorimetric labeling, bioluminescent labeling and/or chemiluminescent labeling. Labeling further may include energy transfer between molecules in the hybridization complex by perturbation analysis, quenching, electron transport between donor and acceptor molecules, the latter of which may be facilitated by double stranded match hybridization complexes. Optionally, if the hybridization complex is unlabeled, detection may be accomplished by measurement of conductance differential between double stranded and non-double stranded DNA. Further, direct detection may be achieved by porous silicon-based optical interferometry or by mass spectrometry. The label may be amplified, and may include for example branched or dendritic DNA. The target DNA may be amplified or not amplified. Further, if the target is amplified and the amplification is an exponential method, it may be, for example, PCR amplified DNA or strand displacement amplification (SDA) amplified DNA. Linear methods of DNA amplification such as rolling circle or transcriptional runoff may also be used.

The present invention provides oligonucleotides that can be used as primers to amplify specific nucleic acid sequences of the *ob* gene. The present invention also provides oligonucleotides that can be used as probes in the detection of amplified specific nucleic acid sequences of the *ob* gene, SEQ ID NO:1 or SEQ ID NO:2. The oligonucleotides can be immobilized on a solid support. Alternatively, a plurality of oligonucleotide probes wherein one or more oligonucleotide probes can be immobilized on an oligonucleotide array.

Among the nucleic acids provided herein are the nucleic acids whose sequence is provided in SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, or a fragment thereof. Additionally, the invention includes mutant or variant nucleic acids of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, or a fragment thereof, any of whose bases may be changed from the corresponding bases shown in SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7, while still hybridizing to the *ob* gene DNA sequence. The invention further includes the complement of the nucleic acid sequence of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7, including fragments, derivatives, analogs and

homologs thereof. The invention additionally includes nucleic acids or nucleic acid fragments, or complements thereto, whose structures include chemical modifications.

The invention also includes an oligonucleotide that includes a portion of the disclosed nucleic acids. Advantageously, the oligonucleotide can be at least 10 nucleotides in length and include at least nine contiguous nucleotides of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7.

As to detection of the hybridization complex formed between probe and target, it is advantageous that the complex is labeled. Typically, in the step of determining hybridization of probe to target, there is a detection of the amount of labeled hybridization complex at the test site or a portion thereof. Any mode or modality of detection consistent with the purpose and functionality of the invention may be utilized, such as optical imaging, electronic imaging, use of charge-coupled devices or other methods of quantification. Labeling may be of the target, capture, or reporter. Various labeling may be by fluorescent labeling, colorimetric labeling or chemiluminescent labeling. In yet another implementation, detection may be via energy transfer between molecules in the hybridization complex. In yet another aspect, the detection may be via fluorescence perturbation analysis. In another aspect the detection may be via conductivity differences between concordant and discordant sites.

In yet another aspect, detection can be carried out using mass spectrometry. In such method, no fluorescent label is necessary. Rather detection is obtained by extremely high levels of mass resolution achieved by direct measurement, for example, by time of flight or by electron spray ionization (ESI).

It is a further object of the present invention to provide such a method that provides to packers increased predictability of carcass grade of livestock purchased. It is a further object of the present invention to provide such a method that allows cow/calf operators to be able to respond to market signals from the feed lot more accurately by producing animals with a greater or lesser genetic predisposition to lay down fat.

Individual animals among assemblies of animals received at feeding facilities are segregated into groups based conventionally on weight and frame type, and additionally based on *ob* genotype. The animals are tested to determine homozygosity or heterozygosity with respect to alleles of the *ob* gene. Animals of such groups will, when maintained together on a uniform diet, exhibit greater body fat condition uniformity at any particular time after such segregation than is exhibited among animals grouped together using current practices.

Individual animals within such a group will attain a desired body condition closer to the time that other individual animals of the same group attain the desired body condition. Such temporal uniformity exceeds that exhibited in groups of otherwise similarly situated animals maintained and fed together using current grouping practices.

5 It will be advantageous to optimize milk production to milk dairy cattle when they are genetically predisposed toward increased milk production (hereinafter TT cattle, i.e., cattle homozygous for the T SNP). Conversely, milking dairy cattle that are less genetically predisposed toward increased milk production (hereinafter CC cattle, i.e., homozygous for the c SNP or CT Cattle, i.e. heterozygous for the SNP) may result in less than optimal milk
10 production.

In another embodiment, it will be advantageous to feed cattle to achieve a high fat grade when they are most genetically predisposed to lay down fat (TT cattle). As to those cattle least genetically predisposed to lay down fat (CC cattle), it will be advantageous to feed these cattle so as to achieve a lower fat grade, or a lean grade, rather than feed them longer to
15 achieve the high fat grade. Those cattle intermediately genetically predisposed to lay down fat (CT cattle), can be fed longer to achieve a high fat grade, or shorter to achieve a lean grade, depending on considerations such as market prices, price trends, feed costs, availability of further feeder cattle to bring into the feed lot, and other like external considerations. On occasion such external considerations may dictate that CC cattle should
20 be fed for a fat grade, however this will most often be so inefficient that such feeding would not be cost effective.

One embodiment of a method of livestock management according to the present invention provides a direct correlation between milk production in livestock animals and the presence of alleles of leptin gene, i.e., *ob*. During the first one hundred days of location, TT
25 animals have the highest milk production. CC animals produce the least amount of milk and CT animals produce an intermediate amount of milk. During the third phase of growth, TT animals also have the highest feed conversion rate, whereas CC animals have the lowest feed conversion rate and CT animals have an intermediate feed conversion rate.

For a ton of feed eaten, TT animals, and particularly TT cattle, will gain the most
30 weight, CC cattle will gain the least weight, and CT cattle will gain an intermediate amount of weight. Thus, for any given number of cattle, the amount of feed eaten per pound of weight gain will decrease as the occurrence of the *ob* T-allele increases. Further, by grouping

cattle according to genotype, as in the method of the present invention, and feeding grouped cattle together, more uniform sized carcasses can be realized since cattle with more similar feed conversion rates will grow in a more similar manner when environmental conditions, such as feed content, are constant. A carcass with a certain minimum level of intramuscular fat will be graded AAA in Canada, corresponding to Choice Grade in the United States. At present such AAA carcasses will bring a premium payment for the feedlot operator.

The invention further comprises a kit for evaluating a sample of livestock DNA. At a minimum, the kit is a container with one or more reagents that identify a polymorphism in the livestock *ob* gene. Advantageously, the reagent is a probe or set of primers that hybridize with the livestock *ob* gene or fragments thereof. Advantageously, the probe is selected from SEQ ID NO:4 and SEQ ID NO:5 or a fragment thereof.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

It is noted that in this disclosure and particularly in the claims, terms such as "comprises", "comprised", "comprising" and the like can have the meaning attributed to it in U.S. Patent law; *e.g.*, they can mean "includes", "included", "including", and the like; and that terms such as "consisting essentially of" and "consists essentially of" have the meaning ascribed to them in U.S. Patent law, *e.g.*, they allow for elements not explicitly recited, but exclude elements that are found in the prior art or that affect a basic or novel characteristic of the invention.

These and other objects, features, and advantages of the invention become further apparent in the following detailed description of the invention when taken in conjunction with the accompanying drawings that illustrate, by way of example, the principles of this invention.

BRIEF DESCRIPTION OF THE DRAWINGS

The following detailed description, given by way of example, but not intended to limit the invention solely to the specific embodiments described, may best be understood in conjunction with the accompanying drawings, in which:

- 5 FIG. 1 depicts non-esterified fatty acid (NEFA)-by leptin and days in milk (DIM),
 FIG. 2 depicts beta-hydroxy butyrate (BHBA)-by leptin,
 FIG. 3 depicts dry matter intake (DMI)-by leptin and DIM and
 FIG. 4 depicts milk yield-by leptin and weeks in milk (WIM).

DETAILED DESCRIPTION

- 10 In the description that follows, a number of terms are extensively utilized. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following terminology is provided:

- An "amplification primer" is an oligonucleotide that is capable of annealing adjacent to a target sequence and serving as an initiation point for DNA synthesis when placed under
15 conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is initiated.

- By "amplifying a segment" as used herein, is meant the production of sufficient multiple copies of the segment to permit relatively facile manipulation of the segment. Manipulation refers to both physical and chemical manipulation, that is, the ability to move
20 bulk quantities of the segment around and to conduct chemical reactions with the segment that result in detectable products. A "segment" of a polynucleotide refers to an oligonucleotide that is a partial sequence of entire nucleotide sequence of the polynucleotide. A "modified segment" refers to a segment in which one or more natural nucleotides have been replaced with one or more modified nucleotides. A "modified, labeled segment refers to
25 a modified segment that also contains a nucleotide, which is different from the modified nucleotide or nucleotides therein, and which is detectably labeled.

- By "analysis" is meant either detection of variance in the nucleotide sequence among two or more related polynucleotides or, in the alternative, the determination of the full nucleotide sequence of a polynucleotide. By "analyzing" the hybridized fragments for an
30 incorporated detectable label identifying the suspected polymorphism is meant that, at some stage of the sequence of events that leads to hybridized fragments, a label is incorporated. The label may be incorporated at virtually any stage of the sequence of events including the

amplification, the cleavage or the hybridization procedures. The label may even be introduced into the sequence of events after cleavage but before hybridization or even after hybridization. The label so incorporated is then observed visually or by instrumental means. The presence of the label identifies the polymorphism due to the fact that the fragments
5 obtained during cleavage are specific to the modified nucleotide(s) used in the amplification and at least one of the modified nucleotide is selected so as to replace a nucleotide involved in the polymorphism.

The term "animal" is used herein to include all vertebrate animals, including humans. It also includes an individual animal in all stages of development, including embryonic and fetal stages. As used herein, the term "production animals" is used interchangeably with
10 "livestock animals" and refers generally to animals raised primarily for food. For example, such animals include, but are not limited to, cattle (bovine), sheep (ovine), pigs (porcine or swine), poultry (avian), and the like. As used herein, the term "cow" or "cattle" is used generally to refer to an animal of bovine origin of any age. Interchangeable terms include
15 "bovine", "calf", "steer", "bull", "heifer" and the like. As used herein, the term "pig" or is used generally to refer to an animal of porcine origin of any age. Interchangeable terms include "piglet", "sow" and the like.

The term "antisense" is intended to refer to polynucleotide molecules complementary to a portion of an RNA marker of the *ob* gene, as defined herein. "Complementary"
20 polynucleotides are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T) in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-
25 methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.

By the term "complementarity" or "complementary" is meant, for the purposes of the specification or claims, a sufficient number in the oligonucleotide of complementary base
30 pairs in its sequence to interact specifically (hybridize) with the target nucleic acid sequence of the *ob* gene polymorphism to be amplified or detected. As known to those skilled in the art, a very high degree of complementarity is needed for specificity and sensitivity involving hybridization, although it need not be 100%. Thus, for example, an oligonucleotide that is

identical in nucleotide sequence to an oligonucleotide disclosed herein, except for one base change or substitution, may function equivalently to the disclosed oligonucleotides. A "complementary DNA" or "cDNA" gene includes recombinant genes synthesized by reverse transcription of messenger RNA ("mRNA").

5 By the term "composition" is meant, for the purposes of the specification or claims, a combination of elements which may include one or more of the following: the reaction buffer for the respective method of enzymatic amplification, plus one or more oligonucleotides specific for *ob* gene polymorphisms, wherein said oligonucleotide is labeled with a detectable moiety.

10 By the terms "consisting essentially of a nucleotide sequence" is meant, for the purposes of the specification or claims, the nucleotide sequence disclosed, and also encompasses nucleotide sequences which are identical except for a one base change or substitution therein.

A "cyclic polymerase-mediated reaction" refers to a biochemical reaction in which a
15 template molecule or a population of template molecules is periodically and repeatedly copied to create a complementary template molecule or complementary template molecules, thereby increasing the number of the template molecules over time.

"Denaturation" of a template molecule refers to the unfolding or other alteration of the structure of a template so as to make the template accessible to duplication. In the case of
20 DNA, "denaturation" refers to the separation of the two complementary strands of the double helix, thereby creating two complementary, single stranded template molecules. "Denaturation" can be accomplished in any of a variety of ways, including by heat or by treatment of the DNA with a base or other denaturant.

A "detectable amount of product" refers to an amount of amplified nucleic acid that
25 can be detected using standard laboratory tools. A "detectable marker" refers to a nucleotide analog that allows detection using visual or other means. For example, fluorescently labeled nucleotides can be incorporated into a nucleic acid during one or more steps of a cyclic polymerase-mediated reaction, thereby allowing the detection of the product of the reaction using, *e.g.* fluorescence microscopy or other fluorescence-detection instrumentation.

30 By the term "detectable moiety" is meant, for the purposes of the specification or claims, a label molecule (isotopic or non-isotopic) which is incorporated indirectly or directly into an oligonucleotide, wherein the label molecule facilitates the detection of the

oligonucleotide in which it is incorporated when the oligonucleotide is hybridized to amplified *ob* gene polymorphisms sequences. Thus, "detectable moiety" is used synonymously with "label molecule". Synthesis of oligonucleotides can be accomplished by any one of several methods known to those skilled in the art. Label molecules, known to those skilled in the art as being useful for detection, include chemiluminescent or fluorescent molecules. Various fluorescent molecules are known in the art which are suitable for use to label a nucleic acid substrate for the method of the present invention. The protocol for such incorporation may vary depending upon the fluorescent molecule used. Such protocols are known in the art for the respective fluorescent molecule.

By "detectably labeled" is meant that a fragment or an oligonucleotide contains a nucleotide that is radioactive, that is substituted with a fluorophore or some other molecular species that elicits a physical or chemical response can be observed by the naked eye or by means of instrumentation such as, without limitation, scintillation counters, colorimeters, UV spectrophotometers and the like. As used herein, a "label" or "tag" refers to a molecule that, when appended by, for example, without limitation, covalent bonding or hybridization, to another molecule, for example, also without limitation, a polynucleotide or polynucleotide fragment, provides or enhances a means of detecting the other molecule. A fluorescence or fluorescent label or tag emits detectable light at a particular wavelength when excited at a different wavelength. A radiolabel or radioactive tag emits radioactive particles detectable with an instrument such as, without limitation, a scintillation counter. Other signal generation detection methods include: chemiluminescence, electrochemiluminescence, raman, colorimetric, hybridization protection assay, and mass spectrometry

"DNA amplification" as used herein refers to any process that increases the number of copies of a specific DNA sequence by enzymatically amplifying the nucleic acid sequence. A variety of processes are known. One of the most commonly used is the polymerase chain reaction (PCR) process of Mullis as described in U.S. Pat. Nos. 4,683,195 and 4,683,202. PCR involves the use of a thermostable DNA polymerase, known sequences as primers, and heating cycles, which separate the replicating deoxyribonucleic acid (DNA), strands and exponentially amplify a gene of interest. Any type of PCR, such as quantitative PCR, RT-PCR, hot start PCR, LAPCR, multiplex PCR, touchdown PCR, *etc.*, may be used. Advantageously, real-time PCR is used. In general, the PCR amplification process involves an enzymatic chain reaction for preparing exponential quantities of a specific nucleic acid

sequence. It requires a small amount of a sequence to initiate the chain reaction and oligonucleotide primers that will hybridize to the sequence. In PCR the primers are annealed to denatured nucleic acid followed by extension with an inducing agent (enzyme) and nucleotides. This results in newly synthesized extension products. Since these newly synthesized sequences become templates for the primers, repeated cycles of denaturing, primer annealing, and extension results in exponential accumulation of the specific sequence being amplified. The extension product of the chain reaction will be a discrete nucleic acid duplex with a termini corresponding to the ends of the specific primers employed.

“DNA” refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA).

By the terms “enzymatically amplify” or “amplify” is meant, for the purposes of the specification or claims, DNA amplification, *i.e.*, a process by which nucleic acid sequences are amplified in number. There are several means for enzymatically amplifying nucleic acid sequences. Currently the most commonly used method is the polymerase chain reaction (PCR). Other amplification methods include LCR (ligase chain reaction) which utilizes DNA ligase, and a probe consisting of two halves of a DNA segment that is complementary to the sequence of the DNA to be amplified, enzyme QB replicase and a ribonucleic acid (RNA) sequence template attached to a probe complementary to the DNA to be copied which is used to make a DNA template for exponential production of complementary RNA; strand displacement amplification (SDA); QB replicase amplification (QBRA); self-sustained replication (3SR); and NASBA (nucleic acid sequence-based amplification), which can be performed on RNA or DNA as the nucleic acid sequence to be amplified.

The “extension of the primer molecules” refers to the addition of nucleotides to a primer molecule so as to synthesize a nucleic acid complementary to a template molecule. “Extension of the primer molecules” does not necessarily imply that the primer molecule is

extended to synthesize a complete complementary template molecule. Rather, even if only a fraction of the template molecule has been copied, the primer is still considered extended.

A "fragment" of a molecule such as a protein or nucleic acid is meant to refer to any portion of the amino acid or nucleotide genetic sequence.

5 As used herein, "fluorescence resonance energy transfer pair" or "FRET pair" refers to a pair of fluorophores comprising a donor fluorophore and acceptor fluorophore, wherein the donor fluorophore is capable of transferring resonance energy to the acceptor fluorophore. In other words the emission spectrum of the donor fluorophore overlaps the absorption spectrum of the acceptor fluorophore. In advantageous fluorescence resonance energy
10 transfer pairs, the absorption spectrum of the donor fluorophore does not substantially overlap the absorption spectrum of the acceptor fluorophore. As used herein, "a donor oligonucleotide probe" refers to an oligonucleotide that is labeled with a donor fluorophore of a fluorescent resonance energy transfer pair. As used herein, "an acceptor oligonucleotide probe" refers to an oligonucleotide that is labeled with an acceptor fluorophore of a
15 fluorescent resonance energy transfer pair. As used herein, "FRET oligonucleotide pair" refers to the donor oligonucleotide probe and the acceptor oligonucleotide probe pair that form a fluorescence resonance energy transfer relationship when the donor oligonucleotide probe and the acceptor oligonucleotide probe are both hybridized to their complementary target nucleic acid sequences. Two separate FRET oligonucleotide pairs, each specific for
20 one locus and each comprising a different acceptor dye may be used at the same time. Acceptable fluorophore pairs for use as fluorescent resonance energy transfer pairs are well known to those skilled in the art and include, but are not limited to, fluorescein/rhodamine, phycoerythrin/Cy7, fluorescein/Cy5, fluorescein/Cy5.5, fluorescein/LC Red 640, and fluorescein/LC Red 705.

25 A "functional derivative" of a sequence, either protein or nucleic acid, is a molecule that possesses a biological activity (either functional or structural) that is substantially similar to a biological activity of the protein or nucleic acid sequence. A functional derivative of a protein may or may not contain post-translational modifications such as covalently linked carbohydrate, depending on the necessity of such modifications for the performance of a
30 specific function. The term "functional derivative" is intended to include the "fragments," "segments," "variants," "analogs," or "chemical derivatives" of a molecule.

As used herein, the term "genome" refers to all the genetic material in the chromosomes of a particular organism. Its size is generally given as its total number of base pairs. Within the genome, the term "gene" refers to an ordered sequence of nucleotides located in a particular position on a particular chromosome that encodes specific functional product (e.g., a protein or RNA molecule). For example, it is known that the protein leptin is encoded by the *ob* (*obese*) gene and appears to be involved in the regulation of appetite, basal metabolism and fat deposition. In general, an animal's genetic characteristics, as defined by the nucleotide sequence of its genome, are known as its "genotype," while the animal's physical traits are described as its "phenotype."

By "heterozygous" or "heterozygous polymorphism" is meant that the two alleles of a diploid cell or organism at a given locus are different, that is, that they have a different nucleotide exchanged for the same nucleotide at the same place in their sequences.

By "homozygous" is meant that the two alleles of a diploid cell or organism at a given locus are identical, that is, that they have the same nucleotide for nucleotide exchange at the same place in their sequences.

By "hybridization" or "hybridizing," as used herein, is meant the formation of A-T and C-G base pairs between the nucleotide sequence of a fragment of a segment of a polynucleotide and a complementary nucleotide sequence of an oligonucleotide. By complementary is meant that at the locus of each A, C, G or T (or U in a ribonucleotide) in the fragment sequence, the oligonucleotide sequenced has a T, G, C or A, respectively. The hybridized fragment/oligonucleotide is called a "duplex."

A "hybridization complex", such as in a sandwich assay, means a complex of nucleic acid molecules including at least the target nucleic acid and sensor probe. It may also include an anchor probe.

By "immobilized on a solid support" is meant that a fragment, primer or oligonucleotide is attached to a substance at a particular location in such a manner that the system containing the immobilized fragment, primer or oligonucleotide may be subjected to washing or other physical or chemical manipulation without being dislodged from that location. A number of solid supports and means of immobilizing nucleotide-containing molecules to them are known in the art; any of these supports and means may be used in the methods of this invention.

As used herein, the term "increased weight gain" means a biologically significant increase in weight gain above the mean of a given population.

As used herein, the term "locus" or "loci" refers to the site of a gene on a chromosome. Pairs of genes, known as "alleles" control the hereditary trait produced by a gene locus. Each animal's particular combination of alleles is referred to as its "genotype". Where both alleles are identical the individual is said to be homozygous for the trait controlled by that gene pair; where the alleles are different, the individual is said to be heterozygous for the trait.

A "melting temperature" is meant the temperature at which hybridized duplexes dehybridize and return to their single-stranded state. Likewise, hybridization will not occur in the first place between two oligonucleotides, or, herein, an oligonucleotide and a fragment, at temperatures above the melting temperature of the resulting duplex. It is presently advantageous that the difference in melting point temperatures of oligonucleotide-fragment duplexes of this invention be from about 1°C to about 10°C so as to be readily detectable.

As used herein, the term "nucleic acid molecule" is intended to include DNA molecules (*e.g.*, cDNA or genomic DNA), RNA molecules (*e.g.*, mRNA), analogs of the DNA or RNA generated using nucleotide analogs, and derivatives, fragments and homologs thereof. The nucleic acid molecule can be single-stranded or double-stranded, but advantageously is double-stranded DNA. An "isolated" nucleic acid molecule is one that is separated from other nucleic acid molecules that are present in the natural source of the nucleic acid. A "nucleoside" refers to a base linked to a sugar. The base may be adenine (A), guanine (G) (or its substitute, inosine (I)), cytosine (C), or thymine (T) (or its substitute, uracil (U)). The sugar may be ribose (the sugar of a natural nucleotide in RNA) or 2-deoxyribose (the sugar of a natural nucleotide in DNA). A "nucleotide" refers to a nucleoside linked to a single phosphate group.

As used herein, the term "oligonucleotide" refers to a series of linked nucleotide residues, which oligonucleotide has a sufficient number of nucleotide bases to be used in a PCR reaction. A short oligonucleotide sequence may be based on, or designed from, a genomic or cDNA sequence and is used to amplify, confirm, or reveal the presence of an identical, similar or complementary DNA or RNA in a particular cell or tissue. Oligonucleotides may be chemically synthesized and may be used as primers or probes.

Oligonucleotide means any nucleotide of more than 3 bases in length used to facilitate detection or identification of a target nucleic acid, including probes and primers.

“Polymerase chain reaction” or “PCR” refers to a thermocyclic, polymerase-mediated, DNA amplification reaction. A PCR typically includes template molecules, oligonucleotide primers complementary to each strand of the template molecules, a thermostable DNA polymerase, and deoxyribonucleotides, and involves three distinct processes that are multiply repeated to effect the amplification of the original nucleic acid. The three processes (denaturation, hybridization, and primer extension) are often performed at distinct temperatures, and in distinct temporal steps. In many embodiments, however, the hybridization and primer extension processes can be performed concurrently. The nucleotide sample to be analyzed may be PCR amplification products provided using the rapid cycling techniques described in U.S. Pat. Nos. 6,569,672; 6,569,627; 6,562,298; 6,556,940; 6,569,672; 6,569,627; 6,562,298; 6,556,940; 6,489,112; 6,482,615; 6,472,156; 6,413,766; 6,387,621; 6,300,124; 6,270,723; 6,245,514; 6,232,079; 6,228,634; 6,218,193; 6,210,882; 6,197,520; 6,174,670; 6,132,996; 6,126,899; 6,124,138; 6,074,868; 6,036,923; 5,985,651; 5,958,763; 5,942,432; 5,935,522; 5,897,842; 5,882,918; 5,840,573; 5,795,784; 5,795,547; 5,785,926; 5,783,439; 5,736,106; 5,720,923; 5,720,406; 5,675,700; 5,616,301; 5,576,218 and 5,455,175, the disclosures of which are incorporated by reference in their entireties. Other methods of amplification include, without limitation, NASBR, SDA, 3SR, TSA and rolling circle replication. It is understood that, in any method for producing a polynucleotide containing given modified nucleotides, one or several polymerases or amplification methods may be used. The selection of optimal polymerization conditions depends on the application.

A “polymerase” is an enzyme that catalyzes the sequential addition of monomeric units to a polymeric chain, or links two or more monomeric units to initiate a polymeric chain. In advantageous embodiments of this invention, the “polymerase” will work by adding monomeric units whose identity is determined by and which is complementary to a template molecule of a specific sequence. For example, DNA polymerases such as DNA pol I and Taq polymerase add deoxyribonucleotides to the 3' end of a polynucleotide chain in a template-dependent manner, thereby synthesizing a nucleic acid that is complementary to the template molecule. Polymerases may be used either to extend a primer once or repetitively or to amplify a polynucleotide by repetitive priming of two complementary strands using two primers.

A "polynucleotide" refers to a linear chain of nucleotides connected by a phosphodiester linkage between the 3'-hydroxyl group of one nucleoside and the 5'-hydroxyl group of a second nucleoside which in turn is linked through its 3'-hydroxyl group to the 5'-hydroxyl group of a third nucleoside and so on to form a polymer comprised of nucleosides
5 linked by a phosphodiester backbone. A "modified polynucleotide" refers to a polynucleotide in which one or more natural nucleotides have been partially or substantially completely replaced with modified nucleotides.

A "primer" is a short oligonucleotide, the sequence of which is complementary to a segment of the template which is being replicated, and which the polymerase uses as the
10 starting point for the replication process. By "complementary" is meant that the nucleotide sequence of a primer is such that the primer can form a stable hydrogen bond complex with the template; *i.e.*, the primer can hybridize to the template by virtue of the formation of base-pairs over a length of at least ten consecutive base pairs.

The primers herein are selected to be "substantially" complementary to different
15 strands of a particular target DNA sequence. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary
20 bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to hybridize therewith and thereby form the template for the synthesis of the extension product.

"Probes" refer to nucleic acid sequences of variable length, used in the detection of identical, similar, or complementary nucleic acid sequences by hybridization. An
25 oligonucleotide sequence used as a detection probe may be labeled with a detectable moiety. Various labeling moieties are known in the art. Said moiety may, for example, either be a radioactive compound, a detectable enzyme (*e.g.* horse radish peroxidase (HRP)) or any other moiety capable of generating a detectable signal such as a calorimetric, fluorescent, chemiluminescent or electrochemiluminescent signal. The detectable moiety may be
30 detected using known methods. In one embodiment the probe oligomers are generally 8 to 44-mers and advantageously about 10 to 12-mers and advantageously about 11-mers.

As used herein, the term "protein" refers to a large molecule composed of one or more chains of amino acids in a specific order. The order is determined by the base sequence of nucleotides in the gene coding for the protein. Proteins are required for the structure, function, and regulation of the body's cells, tissues, and organs. Each protein has a unique function.

As used herein, the terms "quality traits" or "physical characteristics" refer to advantageous properties of the animal resulting from genetics. Quality traits include, but are not limited to, the animal's genetic ability to metabolize energy, produce milk, put on intramuscular fat, lay eggs, produce offspring, produce particular proteins in meat or milk, or retain protein in milk. Physical characteristics include marbled or lean meats. The terms are used interchangeably.

A "restriction enzyme" refers to an endonuclease (an enzyme that cleaves phosphodiester bonds within a polynucleotide chain) that cleaves DNA in response to a recognition site on the DNA. The recognition site (restriction site) consists of a specific sequence of nucleotides typically about 4-8 nucleotides long.

A "single nucleotide polymorphism" or "SNP" refers to polynucleotide that differs from another polynucleotide by a single nucleotide exchange. For example, without limitation, exchanging one A for one C, G or T in the entire sequence of polynucleotide constitutes a SNP. Of course, it is possible to have more than one SNP in a particular polynucleotide. For example, at one locus in a polynucleotide, a C may be exchanged for a T, at another locus a G may be exchanged for an A and so on. When referring to SNPs, the polynucleotide is most often DNA and the SNP is one that usually results in a deleterious change in the genotype of the organism in which the SNP occurs.

As used herein, a "template" refers to a target polynucleotide strand, for example, without limitation, an unmodified naturally-occurring DNA strand, which a polymerase uses as a means of recognizing which nucleotide it should next incorporate into a growing strand to polymerize the complement of the naturally-occurring strand. Such DNA strand may be single-stranded or it may be part of a double-stranded DNA template. In applications of the present invention requiring repeated cycles of polymerization, *e.g.*, the polymerase chain reaction (PCR), the template strand itself may become modified by incorporation of modified nucleotides, yet still serve as a template for a polymerase to synthesize additional polynucleotides.

A "thermocyclic reaction" is a multi-step reaction wherein at least two steps are accomplished by changing the temperature of the reaction.

A "thermostable polymerase" refers to a DNA or RNA polymerase enzyme that can withstand extremely high temperatures, such as those approaching 100°C. Often, 5 thermostable polymerases are derived from organisms that live in extreme temperatures, such as *Thermus aquaticus*. Examples of thermostable polymerases include Taq, Tth, Pfu, Vent, deep vent, ULTma, and variations and derivatives thereof.

A "variance" is a difference in the nucleotide sequence among related polynucleotides. The difference may be the deletion of one or more nucleotides from the 10 sequence of one polynucleotide compared to the sequence of a related polynucleotide, the addition of one or more nucleotides or the substitution of one nucleotide for another. The terms "mutation," "polymorphism" and "variance" are used interchangeably herein. As used herein, the term "variance" in the singular is to be construed to include multiple variances; i.e., two or more nucleotide additions, deletions and/or substitutions in the same 15 polynucleotide. A "point mutation" refers to a single substitution of one nucleotide for another.

A typical growth curve is correlated with the weight of production animals. Present production practices vary among the specific industries as to the point on the curve at which the animal is considered ready for slaughter. For poultry and pigs, for example, present 20 practice is to slaughter near the beginning of phase three, where the growth curve begins to flatten. At this portion of the curve, the amount of time and feed required to produce a pound of gain increase, so economics dictates that the animal should be slaughtered at that time and replaced in the feeding facility with an animal in the second phase where weight gain is much more rapid and efficient in terms of feed conversion.

25 The growth curve can also be correlated with milk production in dairy cattle.

Following the successful live birth of a heifer calf, the dairy farmer begins to manage that animal toward the goal of successfully breeding that animal at 15 months of age. The heifer must be quickly and effectively moved through a diet of milk replacer and on to roughage diets to ensure that she attains a target weight gain. In the case of Holsteins, she 30 must reach a minimum weight of 800 lbs when she is successfully bred at 15 months of age.

During the early phase of life, the heifer is subject to illness and so a fully functional immune system is very important. Leptin has been shown to have a moderating influence on

elements of the inflammatory response and can play a role in an immune function (Houseknecht). Leptin genotype, and the resulting changes in structure and function of the protein, can influence the effectiveness of the protein in moderating immune function.

5 Attainment of appropriate growth during the time period from birth to 15 months of age is required to ensure the heifer will make her target weight. Leptin has been shown to influence growth rate and carcass composition in growing cattle (Geary) and the effect of the leptin genotype on body composition in cattle is well described in previous patent applications.

10 As the animal grows, it will eventually attain puberty. Leptin has a powerful influence on reproduction (Margetic; Williams) and puberty (Amstalden; Baker). Alterations in the central or peripheral control of reproduction via changes in leptin function resulting from the genotype differences has the potential to significantly influence the attainment of puberty and successful breeding in heifers.

15 In the time period up to 15 months, and from 15 months through to calving, the heifer must develop a normal udder. Leptin is present in fat tissue in the udder and normal development of the udder - required for proper lactation performance - is affected by leptin and will likely be affected by leptin genotype (Silva; Chilliard).

20 The time period from calving through to peak lactation (approximately the first 100 days of lactation), is the most stressful period in the life of the dairy cow. That animal must move quickly from essentially a roughage diet to one which is high in energy value (concentrate) in order that she can meet the huge energy demands of lactation. During this time period, her appetite and daily feed intake is increasing but unable to keep pace with the increased energy demand of lactation and so she falls into negative energy balance. This period of negative energy balance makes her susceptible to metabolic disease and the relationship between leptin, glucose, non-esterified fatty acids and beta-hydroxybutyrate in the regulation of energy production in the post calving dairy cow is extremely important (Delavaud). Recent work from studies examining the relationship between leptin genotype and energy balance in post-calving dairy cows suggest that genotype will have a major impact on energy balance in the post-calving period (Leslie, et al - see attached).

30 A variety of studies have shown the relationship between leptin concentration and milk yield, feed intake, live weight and estrus in dairy cattle (Leifers; Leifers). The ability of the cow to manage the energy demands of lactation, increase her feed consumption and come

back into estrus in order that she can be bred in a timely fashion is extremely challenging. Because leptin is a hormone that is central to regulation of feed intake, the leptin polymorphism will likely influence feed intake, energy balance, milk yield and reproduction, the polymorphism will be shown to be significantly related to all of these events, and
5 management of animals by genotype will be central to efficient dairy production.

Assuming that the cows becomes pregnant, then she must retain the calf during the remainder of her lactation. Approximately 45-60 days prior to her next lactation, the cows is "dried off" and prepared for her next lactation. During late lactation, the cow has returned to positive energy balance and she is beginning to lay down body fat reserves in preparation for
10 her next lactation. It has been shown that the leptin polymorphism will result in alterations in circulating leptin concentration in the period immediately prior to calving which will influence body condition score, feed intake and preparedness for calving and subsequent lactation (Leifers). Managing cows by genotype during this time period, and altering rations as a result will help to ensure that there production can be maximized from all cows.

15 The present invention differs from current practice by using genetic test results to identify, select and group the animals. Rather than rely on a growth curve or visual inspection of animal traits, the present invention allows the farmer to milk and feed livestock according to the individual animal's genetic traits. According to the method of the present invention, it is possible to select a desired trait, such as milk production, identify the
20 polypeptide which specifically encodes for a gene associate with that trait, and genotype animals possessing the associated gene.

Leptin, a 16-kDa adipocyte-specific polypeptide, is encoded by the *ob* (obese) gene and appears to be involved in the regulation of appetite, basal metabolism, fat deposition and milk production. The *ob* gene has been mapped to specific chromosomes in several different
25 animals, allowing the gene to be sequenced in several different species. In the case of leptin, there is significant conservation among the sequences of *ob* DNAs and leptin polypeptides from the tested species. Mutations in the coding sequences of the *ob* gene causing alterations in the amino acid sequence of the leptin polypeptide have been associated with hyperphagia, hypometabolic activity, and excessive fat deposition, i.e., a phenotype characterized by larger
30 body size (a fat phenotype). In the method of the present invention, it is possible to identify the absence or presence of a specific *ob* allele, thus predicting which animals will or will not possess certain carcass characteristics, e.g., increased fat deposition, increased mean fat

deposition, increased percent rib fat, and decreased percent rib lean. For the *ob* gene, the presence of 138-bp allele was positively associated with these characteristics. Thus, bulls homogenous for the 138-bp allele exhibited greater average fat deposition than heterozygous animals.

5 The present invention provides methods wherein the genetic information obtained from individual animals is cross-matched against markers known in the art to predict specific characteristics. A cytosine (C) to thymine (T) transition within an exon (exon 2) of the *ob* gene correspond to an arginine (ARG) to cysteine (CYS) substitution in the leptin polypeptide. The exon 2 polymorphism is a C/T substitution located at position 305 of exon
10 2 of the bovine leptine gene (see, e.g., Buchanan et al. (2002) Genet Sel Evol. 34(1):105-16). Thus, once the genotype of the animal is determined, it is evaluated to determine whether each individual animal possesses the desired trait, i.e., possesses the specific gene. Animals having like genotypes for a specific gene/characteristic are then grouped together. These like-genotype groupings serve as the basis for breeding, feeding, milking and determining
15 slaughter time. Accordingly, the like-genotype groupings provide a more objective method for determining mates for breeding, diets and lengths of feed cycles, milking and slaughter times.

 The individual genotype data of each animal can be recorded and associated with various other data of the animal, e.g. health information, parentage, vaccination history, herd
20 records, and the like. Such information can be forwarded to a government agency to provide traceability of a meat product, or it may serve as the basis for breeding, feeding and marketing information. Once the genotype data is established, and that data may or may not be associated with other data, the data is stored in an accessible database, such as a computer database or a microchip implanted in the animal.

25 Genetic tendencies can be predicted by the results of genotyping. A method and system of the invention comprises tissue sampling, extraction of genetic material from the sampled tissue, molecular genetic analysis of the genetic material, and where the tissue sample is taken from a meat product, comparison of the genotype with known animal genotypes stored on a database. It is contemplated by the methods and systems described
30 herein that the continuity and integrity of each sample is maintained so that the data is accurate and reliable. Steps necessary for ensuring that the data is accurate and reliable are included in the methods and systems taught herein.

Additionally, the method of the present invention contemplates grouping animals according to their genotype in addition to using the phenotype criteria currently employed in feeding, breeding or growing stages practices. For example, in one embodiment of the present invention, feedlot operators who currently group livestock according to size and frame structures, among other phenotypic traits, would use the data obtained from animals' genotypes which correspond to an animal's propensity to exhibit a characteristic associated with the particular gene, and optionally any other associated data, in order to more efficiently manage production. Thus, the feeder is presented with opportunities for considerable efficiencies in livestock production.

Presently, the feeder feeds all his cattle the same, incurring the same costs for each animal, and typically, with excellent management practices, perhaps 40% will receive an optimal grade of Prime, and receive the premium price for the palatability grade. Of these, a significant number will have excess fat and will thus receive a reduced yield grade. The balance of the cattle, 60%, will grade less than Prime, and thus receive a reduced price, although the feed lot costs incurred by the feeder are substantially the same for these cattle receiving the lesser grade. Grouping and feeding the cattle by genotype allows the feeder to treat each group differently with a view to optimizing management strategies and increasing profits.

A tissue sample may be taken from an animal at any time in the lifetime of an animal but before the carcass identity is lost. The tissue sample can comprise hair, including roots, hide, bone, buccal swabs, blood, saliva, milk, semen, embryos, muscle or any internal organs.

The tissue sample is marked with an identifying number or other indicia that relates the sample to the individual animal from which the sample was taken. The identity of the sample advantageously remains constant throughout the methods and systems of the invention thereby guaranteeing the integrity and continuity of the sample during extraction and analysis. Alternatively, the indicia may be changed in a regular fashion that ensures that the data, and any other associated data, can be related back to the animal from which the data was obtained.

The amount/size of sample required is known to those skilled in the art and for example, can be determined by the subsequent steps used in the method and system of the invention and the specific methods of analysis used. Ideally, the size/volume of the tissue sample retrieved should be as consistent as possible within the type of sample and the species

of animal. For example, for cattle, non-limiting examples of sample sizes/methods include non-fatty meat: 0.0002g--0.0010g; hide: 0.0004g--0.0010g; hair roots: greater than five and less than twenty; buccal swabs: 15 to 20 seconds of rubbing with modest pressure in the area between outer lip and gum using one Cytosoft® cytology brush; bone: 0.0020 g--0.0040 g; blood: 30 to 70µL.

Generally, the tissue sample is placed in a container that is labeled using a numbering system bearing a code corresponding to the animal, for example, to the animal's ear tag. Accordingly, the genotype of a particular animal is easily traceable at all times.

The tissue sample is then treated by the desired methods to retrieve the desired data, for example, such as fat content or genotype. Alternatively, the samples can be frozen for preservation and archived, for example, in the factory/slaughterhouse or a central storage location for future extraction/analysis as required.

In an advantageous embodiment of the invention, a sampling device and/or container is supplied to the farmer, a slaughterhouse or retailer. The sampling device advantageously takes a consistent and reproducible sample from individual animals while simultaneously avoiding any cross-contamination of tissue. Accordingly, the size and volume of sample tissues derived from individual animals would be consistent.

In the present invention, a sample of genomic DNA is obtained from a livestock. Generally, hair is used as the source of the DNA. A sufficient amount of cells are obtained to provide a sufficient amount of DNA for analysis. This amount will be known or readily determinable by those skilled in the art. The DNA is isolated from the blood cells by techniques known to those skilled in the art (see, e.g., U.S. Patent Nos. 6,548,256 and 5,989,431, Hirota et al., Jinrui Idengaku Zasshi. 1989 Sep;34(3):217-23 and John et al., Nucleic Acids Res. 1991 Jan 25;19(2):408; the disclosures of which are incorporated by reference in their entireties).

In the method of the present invention, the source of the test nucleic acid is not critical. For example, the test nucleic acid can be obtained from cells within a body fluid of the livestock or from cells constituting a body tissue of the subject. The particular body fluid from which the cells are obtained is also not critical to the present invention. For example, the body fluid may be selected from the group consisting of blood, ascites, pleural fluid and spinal fluid. Furthermore, the particular body tissue from which cells are obtained is also not critical to the present invention. For example, the body tissue may be selected from the group

consisting of skin, endometrial, uterine and cervical tissue. Both normal and tumor tissues can be used. Further, the source of the target material may include RNA or mitochondrial DNA.

The invention further comprises methods of screening livestock to determine those
 5 having predictably increased milk production on based upon the presence or absence of certain polymorphisms in the *ob* gene. In an advantageous embodiment, the *ob* gene polymorphism is a C to T transition that results in an Arg25Cys in the leptin protein.

Any *ob* gene corresponding to the animal of interest can be used to identify the polymorphism(s) of interest in the *ob* gene. The *ob* gene that has been mapped to
 10 chromosome 6 in mice (Friedman & Leibel, 1992, *Cell* 69: 217-220), chromosome 7q31.3 in humans (Isse *et al.*, 1995, *J. Bio. Chem.* 270: 27728-27733) chromosome 4 in cattle (Stone *et al.* 1996, *Mamm. Genome* 7: 399-400), and chromosome 18 in swine (Neuenschwander *et al.*, 1996, *Anim. Genet.* 27: 275-278; Saskai *et al.*, 1996, *Mamm. Genome* 7: 471-471). Sequences have been determined for the *ob* gene from mice (Zhang *et al.*, 1994, *Nature* 372: 425-432), cattle (U.S. Patent No. 6,297,027 to Spurlock), pigs (U.S. Patent No. 6,277,592 and Neuenschwander *et al.*, 1996, *Anim. Genet.* 27: 275-278), and humans (U.S. Patent No. 6,309,857) and there is significant conservation among the sequences of *ob* DNAs and leptin polypeptides from those species (Bidwell *et al.* 1997, *Anim. Endocrinol.* 8: 191-206; Ramsay *et al.* 1998, *J. Anim. Sci.* 76: 484-490).

20 In an advantageous embodiment, the *ob* sequence is a cattle *ob* sequence with the nucleotide sequence

5'TCTGAAGACCTGGATGCGGGTGGTAACGGAGCACGTGGGTGTTCTCGGAGATC
 GACGATGTGCCACGTGTGGTTTCTTCTGTTTTTCAGGCCCCAGAAGCCCATCCCGG
 GAAGGAAAATGCGCTGTGGACCCCTGTATCGATTCCTGTGGCTTTGGCCCTATCT
 25 GTCTTACGTGGAGGCTGTGCCCATCTGCAAGGTCCAGGATGACACCAAACCCCTC
 ATCAAGACAATTGTCACCAGGATCAATGACATCTCACACACGGTAGGGAGGGAC
 TGGGAGACGAGGTAGAACCGTGGCCATCCCGTGGGGGACCCCAGAGGCTGGCGG
 AGGAGGCTGTGCAGCCTTGACAGGGCCCCAGTGGCCTGGACGCCCCCCTGGCA
 TAAAGACAGCTCCTCTCCTCCTCCACTTCCCTTGCCTCCCGCCTTCTCACTCTCCT
 30 CCCTCCCAGACCGGAATCCTAGTGCCAGGCCAGGAGGAGTCACAGAGGTCCT
 GGGGTCCCCTTGGCAGGTGGCCAGAACCCAGCAGCAGTCCCTCTGGGCCTCCAT
 CTCATTCTAGAATGTTTTAGTCGTTAGGCATTCTTCCTGCCTGGTAACTG 3' (SEQ

ID NO:1), which contains the single nucleotide polymorphism at position 189. In another advantageous embodiment, the *ob* sequence is a cattle *ob* sequence with the nucleotide sequence

5' TCTGAAGACCTGGATGCGGGTGGTAACGGAGCACGTGGGTGTTCTCGGAGATC
 5 GACGATGTGCCACGTGTGGTTTCTTCTGTTTTTCAGGCCCCAGAAGCCCATCCCGG
 GAAGGAAAATGCGCTGTGGACCCCTGTATCGATTCCCTGTGGCTTTGGCCCTATCT
 GTCTTACGTGGAGGCTGTGCCCATCCGCAAGGTCCAGGATGACACCAAACCCCTC
 ATCAAGACAATTGTCACCAGGATCAATGACATCTCACACACGGTAGGGAGGGAC
 TGGGAGACGAGGTAGAACCGTGGCCATCCCGTGGGGGACCCAGAGGCTGGCGG
 10 AGGAGGCTGTGCAGCCTTGCACAGGGCCCCAGTGGCCTGGACGCCCCCTGGCA
 TAAAGACAGCTCCTCTCCTCCTCCACTTCCCTTGCCTCCCGCCTTCTCACTCTCCT
 CCCTCCCAGACCGGAATCCTAGTGCCCAGGCCCAGAAGGAGTCACAGAGGTCCT
 GGGGTCCCCTTGGCAGGTGGCCAGAACCCAGCAGCAGTCCCTCTGGGCCTCCAT
 CTCATTTCTAGAATGTTTTAGTCGTTAGGCATTCTTCCTGCCTGGTAACTG 3' (SEQ

15 ID NO:2), which does not contain the single nucleotide polymorphism at position 189. In another embodiment, the bovine *ob* nucleotide sequence can be selected from any one of the sequences corresponding to GenBank Accession Nos. AB003143, AB070368, AB070369, AE003406, AF120500, AF536174, AJ132764, AJ236854, AJ512638, AJ512639, AJ571671, AJ580799, AJ580800, AJ580801, AR171261, AR171262, AR171263, AR171264, AR171265, AY044438, AY138588, NM_000594, NM_000600, NM_000758, NM_173926, NM_173928, NM_174140, NM_174216, NM_180996, U50365, U62385, U65793, U83512 and Y11369.

In an advantageous embodiment, the *ob* sequence is a cattle *ob* sequence with the amino acid sequence

25 MRCGLPLYRFLWLWPYLSYVEAVPIRKVQDDTKTLIKTIVTRINDISHTQSVSSKQRV
 GLDFIPGLHPLLSLSKMDQTLAIYQQILTSLSRNVVQISNDLENLRDLLHLLAASKSC
 PLPQVRALESLESLGVVLEASLYSTEVVALSRLQGSLLQDMLRQLDLSPGC (SEQ ID
 NO:3). In another embodiment, the bovine *ob* amino acid sequence can be selected from any one of the sequences corresponding to Entrez Protein Accession Nos. AAE82807, AAK95823, AAN04050, AAN28921, BAA19750, BAB63371, CAA72197, CAB38018, CAB64255, CAD54745, CAE45337, CAE45338, CAE45339, NP_000585, NP_000591,
 30

NP_000749, NP_776351, NP_776353, NP_776565, NP_776641, NP_851339, P50595 and Q9BEG9, the disclosures of which are incorporated by reference in their entireties.

5 In an embodiment wherein the *ob* sequence is an ovine *ob* sequence, the ovine *ob* nucleotide sequence can be selected from any one of the sequences corresponding to GenBank Accession Nos. AF310264, AF118636 and U63719 and the ovine *ob* amino acid sequence can be selected from any one of the sequences corresponding to Entrez Protein Accession Nos. AAB51695, AAD17249, P79211, Q28602 and Q28603, the disclosures of which are incorporated by reference in their entireties.

10 In an embodiment wherein the *ob* sequence is an avian *ob* sequence, the avian *ob* nucleotide sequence can be selected from any one of the sequences corresponding to GenBank Accession Nos. NM_012614, NT_032977 and NW_047717 and the avian *ob* amino acid can be selected from the sequence corresponding to Entrez Protein Accession No. NP_036746, the disclosures of which are incorporated by reference in their entireties.

15 In an embodiment wherein the *ob* sequence is an swine *ob* sequence, the swine *ob* nucleotide sequence can be selected from any one of the sequences corresponding to GenBank Accession Nos. AF026976, AF036908, AF052691, AF092422, AF102856, AF167719, AF184172, AF184173, AF477386, AF477387, AH009271, AH011524, AJ223162, AJ223163, AY008846, AY079082, AY079083, U40812, U59894, U63540, U66254, U67739 and U72070 and the swine *ob* amino acid sequence can be selected from
20 any one of the sequences corresponding to Entrez Protein Accession Nos. AAB06579, AAB40624, AAB61244, AAB62399, AAD23567, AAK95823, AAN04050, AAN28921, BAA19750, BAB63371, CAA72197, CAB38018, CAB64255, CAD54745, CAE45337, CAE45338, CAE45339, NP_776351, NP_776353, NP_776565, NP_776641, NP_851339, P50595 and Q9BEG9, the disclosures of which are incorporated by reference in their
25 entireties.

Also disclosed herein are oligonucleotides that can be used as primers to amplify specific nucleic acid sequences of the *ob* gene. The present invention also provides oligonucleotides that can be used as probes in the detection of amplified specific nucleic acid sequences of the *ob* gene. In certain embodiments, these probes and primers consist of
30 oligonucleotide fragments. Such fragments should be of sufficient length to provide specific hybridization to an RNA or DNA tissue sample. The sequences typically will be about 8 to

about 44 nucleotides, but may be longer. Longer sequences, *e.g.*, from about 14 to about 50, are advantageous for certain embodiments.

Nucleic acid molecules having contiguous stretches of about 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 nucleotides from a sequence selected from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:7 are contemplated. Molecules that are complementary to the above mentioned sequences and that bind to these sequences under high stringency conditions also are contemplated. These probes will be useful in a variety of hybridization embodiments, such as Southern and Northern blotting. In some cases, it is contemplated that probes may be used that hybridize to multiple target sequences without compromising their ability to effectively detect the *ob* gene.

Various probes and primers can be designed around the disclosed nucleotide sequences. Primers may be of any length but, typically, are about 10 to about 24 bases in length. A probe or primer can be any stretch of at least 8, advantageously at least 10, more advantageously at least 12, 13, 14, or 15, such as at least 20, *e.g.*, at least 23 or 25, for instance at least 27 or 30 nucleotides. As to PCR or hybridization primers or probes and optimal lengths therefor, reference is also made to Kajimura et al., GATA 7(4):71-79 (1990), the disclosure of which is incorporated by reference in its entirety. In certain embodiments, it is contemplated that multiple probes may be used for hybridization to a single sample. Designing and testing the probes and primers around the *ob* nucleotide sequences described above and from any one of the sequences corresponding to the accession numbers listed can be accomplished by one of ordinary skill in the art.

The use of a hybridization probe of between 10 and 30 nucleotides in length allows the formation of a duplex molecule that is both stable and selective. Molecules having complementary sequences over stretches greater than 12 bases in length are generally advantageous, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of particular hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having stretches of 16 to 24 nucleotides, or even longer where desired. Such fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means or by introducing selected sequences into recombinant vectors for recombinant production.

Methods for making a vector or recombinants or plasmid for amplification of the fragment either *in vivo* or *in vitro* can be any desired method, *e.g.*, a method which is by or

analogous to the methods disclosed in, or disclosed in documents cited in: U.S. Patent Nos. 4,603,112; 4,769,330; 4,394,448; 4,722,848; 4,745,051; 4,769,331; 4,945,050; 5,494,807; 5,514,375; 5,744,140; 5,744,141; 5,756,103; 5,762,938; 5,766,599; 5,990,091; 5,174,993; 5,505,941; 5,338,683; 5,494,807; 5,591,639; 5,589,466; 5,677,178; 5,591,439; 5,552,143; 5,580,859; 6,130,066; 6,004,777; 6,130,066; 6,497,883; 6,464,984; 6,451,770; 6,391,314; 6,387,376; 6,376,473; 6,368,603; 6,348,196; 6,306,400; 6,228,846; 6,221,362; 6,217,883; 6,207,166; 6,207,165; 6,159,477; 6,153,199; 6,090,393; 6,074,649; 6,045,803; 6,033,670; 6,485,729; 6,103,526; 6,224,882; 6,312,682; 6,348,450 and 6; 312,683; U.S. patent application Serial No. 920,197, filed October 16, 1986; WO 90/01543; W091/11525; WO 94/16716; WO 96/39491; WO 98/33510; EP 265785; EP 0 370 573; Andreansky et al., Proc. Natl. Acad. Sci. USA 1996;93:11313-11318; Ballay et al., EMBO J. 1993;4:3861-65; Felgner et al., J. Biol. Chem. 1994;269:2550-2561; Frolov et al., Proc. Natl. Acad. Sci. USA 1996;93:11371-11377; Graham, Tibtech 1990;8:85-87; Grunhaus et al., Sem. Virol. 1992;3:237-52; Ju et al., Diabetologia 1998;41:736-739; Kitson et al., J. Virol. 1991;65:3068-3075; McClements et al., Proc. Natl. Acad. Sci. USA 1996;93:11414-11420; Moss, Proc. Natl. Acad. Sci. USA 1996;93:11341-11348; Paoletti, Proc. Natl. Acad. Sci. USA 1996;93:11349-11353; Pennock et al., Mol. Cell. Biol. 1984;4:399-406; Richardson (Ed), Methods in Molecular Biology 1995;39, "Baculovirus Expression Protocols," Humana Press Inc.; Smith et al. (1983) Mol. Cell. Biol. 1983;3:2156-2165; Robertson et al., Proc. Natl. Acad. Sci. USA 1996;93:11334-11340; Robinson et al., Sem. Immunol. 1997;9:271; and Roizman, Proc. Natl. Acad. Sci. USA 1996;93:11307-11312.

Accordingly, the nucleotide sequences of the invention may be used for their ability to selectively form duplex molecules with complementary stretches of genes or RNAs or to provide primers for amplification of DNA or RNA from tissues. Depending on the application envisioned, one will desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of probe towards target sequence.

For applications requiring high selectivity, one will typically desire to employ relatively stringent conditions to form the hybrids, *e.g.*, one will select relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.10 M NaCl at temperatures of about 50° C to about 70° C. Such high stringency conditions tolerate little, if any, mismatch between the probe and the template or target strand, and would be particularly suitable for isolating specific genes or detecting specific mRNA transcripts. It is

generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide.

It will be understood that this invention is not limited to the particular probes disclosed herein and particularly is intended to encompass at least nucleic acid sequences that are hybridizable to the disclosed sequences or are functional sequence analogs of these sequences.

One embodiment of the present invention is directed to a nucleic acid sequences (oligonucleotides) useful as primers and/or probes in the detection of an *ob* gene polymorphism in specimens. Also, the present invention is directed to a method of detecting the presence of *ob* gene polymorphism in a specimen wherein the oligonucleotides of the present invention may be used to amplify target nucleic acid sequences of an *ob* gene polymorphism that may be contained within a livestock specimen, and/or to detect the presence or absence of amplified target nucleic acid sequences of the *ob* gene polymorphism. Respective oligonucleotides may be used to amplify and/or detect *ob* gene and *ob* gene nucleic acid sequences. By using the oligonucleotides of the present invention and according to the methods of the present invention, as few as one to ten copies of the *ob* gene polymorphism may be detected in the presence of milligram quantities of extraneous DNA.

One embodiment of the present invention is directed to *ob* gene-specific oligonucleotides that can be used to amplify sequences of *ob* gene DNA, and to subsequently determine if amplification has occurred, from DNA extracted from a livestock specimen. A pair of *ob* gene-specific DNA oligonucleotide primers are used to hybridize to *ob* gene genomic DNA that may be present in DNA extracted from a livestock specimen, and to amplify the specific segment of genomic DNA between the two flanking primers using enzymatic synthesis and temperature cycling. Each pair of primers are designed to hybridize only to the *ob* gene DNA to which they have been synthesized to complement; one to each strand of the double-stranded DNA. Thus, the reaction is specific even in the presence of microgram quantities of heterologous DNA. For the purposes of this description, the primer derived from the sequence of the positive strand of DNA will be referred to as the "positive (+) primer", and the primer derived from the sequence of the negative strand will be referred to as the "negative (-) primer". Sequences that may be used include the primers AGGGATGCCTGGACACAAGA (sense, SEQ ID NO:4) and ATTGCCACCACCAGCAGCACCA (antisense, SEQ ID NO:5) and the probes

CATCTGCTATGCGAATGCTTTG (SEQ ID NO:6) and GCTAATTATATTGTAAGACA (SEQ ID NO:7).

5 In one embodiment, the present invention relates to a composition for the detection of *ob* gene polymorphisms, consisting essentially of at least one purified and isolated oligonucleotide consisting of a nucleic acid sequence which complements and specifically hybridizes to an *ob* gene nucleic acid molecule, wherein said sequence is selected from the group consisting of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:7, and a nucleotide sequence which differs from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:7, by a one base change or substitution therein.

10 In another embodiment, the present invention relates to a method of detecting the presence of an *ob* gene polymorphism in a sample comprising (a) contacting the sample with the above-described nucleic acid probe, under conditions such that hybridization occurs, and (b) detecting the presence of the probe bound to the DNA segment. In an advantageous embodiment, the *ob* gene polymorphism is a C to T transition that results in an Arg25Cys in the leptin protein.

15 In another embodiment, the present invention relates to a method of detecting the presence of an *ob* gene polymorphism in a sample comprising (a) contacting the sample with the above-described nucleic acid probe, under conditions such that hybridization occurs, and (b) detecting the presence of the probe bound to the DNA segment. In an advantageous embodiment, the *ob* gene polymorphism is a C to T transition that results in an Arg25Cys in the leptin protein.

20 The actual hybridization reaction represents one of the most important and central steps in the whole process. The hybridization step involves placing the prepared DNA sample in contact with a specific sensor probe at set optimal conditions for hybridization to occur between the target DNA sequence and probe.

25 In their most basic form, hybridization assays function by discriminating oligonucleotide probe sensors against matched and mismatched targets. Currently, a variety of methods are available for detection and analysis of the hybridization events. Depending on the sensor group (fluorophore, enzyme, radioisotope, etc.) used to label the DNA probe, detection and analysis are carried out fluorimetrically, colorimetrically, or by
30 autoradiography. By observing and measuring emitted radiation, such as fluorescent radiation or particle emission, information may be obtained about the hybridization events.

The secondary and tertiary structure of a single stranded target nucleic acid may be affected by binding "helper" oligonucleotides in addition to "probe" oligonucleotides causing a higher T_m to be exhibited between the probe and target nucleic acid.

Methods are provided for the analysis and determination of SNPs in a genetic target.

5 In this embodiment, both wild type and mutant alleles are distinguished, if present in a sample, at a single capture site by detecting the presence of hybridized allele-specific probes labeled with fluorophores sensitive to excitation at various wave lengths.

In one embodiment, a target nucleic acid is first amplified, such as by PCR or SDA. The amplified dsDNA product is then denatured and hybridized with a probe. The

10 hybridization complex formed is then subjected to destabilizing conditions to differentiate and determination of the *ob* SNP.

In another embodiment, the present invention relates to a method of detecting the presence of an *ob* gene polymorphism in a sample comprising a) contacting the sample with the above-described nucleic acid probe, under conditions such that hybridization occurs, b)

15 enzymatically amplifying a specific region of the *ob* gene nucleic acid molecules, and c) detecting the presence of the probe bound to the DNA segment. In an advantageous embodiment, the *ob* gene polymorphism is a C to T transition that results in an Arg25Cys in the leptin protein.

In another embodiment, the present invention relates to a method of detecting the presence of an *ob* gene polymorphism in a sample comprising a) contacting the sample with

20 the oligonucleotide primer pair of SEQ ID NO:4 and SEQ ID NO:5 that under suitable conditions permitting hybridization of the oligonucleotides to the nucleic acid molecules of the *ob* gene, b) enzymatically amplifying a specific region of the *ob* gene nucleic acid molecules using the oligonucleotide pair of SEQ ID NO:4 and SEQ ID NO:5 to form nucleic

25 acid amplification products, c) contacting the amplified target sequences from step be, is present, with hybridization probes comprising the oligonucleotide pair of SEQ ID NO:6 and SEQ ID NO:7, labeled with a detectable moiety under suitable conditions permitting hybridization of the labeled oligonucleotide probe to amplified target sequences, and d)

30 detecting the presence of amplified target sequences by detecting the detectable moiety of the labeled oligonucleotide probe hybridized to amplified target sequences. In an advantageous embodiment, prior to performing the above method, the sample is treated to release nucleic acid molecules from cells in the sample. In another advantageous embodiment, the presence

of the amplified target sequences hybridized labeled oligonucleotide probe correlates to the presence of an *ob* gene polymorphism in the sample. In an advantageous embodiment, the *ob* gene polymorphism is a C to T transition that results in an Arg25Cys in the leptin protein.

Any one of the methods commercially available may accomplish amplification of
5 DNA. For example, the polymerase chain reaction may be used to amplify the DNA. Once the primers have hybridized to opposite strands of the target DNA, the temperature is raised to permit replication of the specific segment of DNA across the region between the two primers by a thermostable DNA polymerase. Then the reaction is thermocycled so that at each cycle the amount of DNA representing the sequences between the two primers is
10 doubled, and specific amplification of the *ob* gene DNA sequences, if present, results.

Further identification of the amplified DNA fragment, as being derived from *ob* gene DNA, may be accomplished by liquid hybridization. This method utilizes one or more oligonucleotides labeled with detectable moiety as probes to specifically hybridize to the amplified segment of *ob* gene DNA. Detection of the presence of sequence-specific
15 amplified *ob* gene DNA may be accomplished by simultaneous detection of the complex comprising the labeled oligonucleotide hybridized to the sequence-specific amplified *ob* gene DNA ("amplified target sequences") with respect to the DNA amplification. Detection of the presence of sequence-specific amplified *ob* gene DNA may also be accomplished using a gel retardation assay with subsequent detection of the complex comprising the labeled
20 oligonucleotide hybridized to the sequence-specific amplified *ob* gene DNA.

In such a enzymatic amplification reaction hybridization system of *ob* gene allele detection, a specimen of blood, CSF, amniotic fluid, urine, body secretions, or other body fluid is subjected to a DNA extraction procedure. High molecular weight DNA may be purified from blood cells, tissue cells, or virus particles (collectively referred to herein as
25 "cells") contained in the livestock specimen using proteinase (proteinase K) extraction and ethanol precipitation. DNA may be extracted from a livestock specimen using other methods known in the art. Then, for example, the DNA extracted from the livestock specimen is enzymatically amplified in the polymerase chain reaction using *ob* gene-specific oligonucleotides (SEQ ID NO:4 and SEQ ID NO:5) as primer pairs. Following
30 amplification, *ob* gene-specific oligonucleotides (SEQ ID NO:6 and SEQ ID NO:7) labeled with an appropriate detectable label are hybridized to the amplified target sequences, if present.

The contents of the hybridization reaction are then analyzed for detection of the sequence-specific amplified *ob* gene DNA, if present in the DNA extracted from the livestock specimen. Thus, the oligonucleotides of the present invention have commercial applications in diagnostic kits for the detection of *ob* gene DNA in livestock specimens.

5 The test samples suitable for nucleic acid probing methods of the present invention include, for example, cells or nucleic acid extracts of cells, or biological fluids. The sample used in the above-described methods will vary based on the assay format, the detection method and the nature of the tissues, cells or extracts to be assayed. Methods for preparing nucleic acid extracts of cells are well known in the art and can be readily adapted in order to
10 obtain a sample that is compatible with the method utilized.

 In a related embodiment of the present invention, the *ob* gene-specific oligonucleotides may be used to amplify and detect *ob* gene polymorphisms from DNA extracted from a livestock specimen. In this embodiment, the oligonucleotides used as primers may be labeled directly with detectable moiety, or synthesized to incorporate the
15 label molecule. Depending on the label molecule used, the amplification products can then be detected, for example, after binding onto an affinity matrix, using isotopic or calorimetric detection. In an advantageous embodiment, the *ob* gene polymorphism is a C to T transition that results in an Arg25Cys in the leptin protein.

 In an advantageous embodiment of this invention, cyclic polymerase-mediated
20 reactions are performed. In certain embodiments of this invention, these processes are accomplished by changing the temperature of the solution containing the templates, primers, and polymerase. In such embodiments, the denaturation step is typically accomplished by shifting the temperature of the solution to a temperature sufficiently high to denature the template. In some embodiments, the hybridization step and the extension step are performed
25 at different temperatures. In other embodiments, however, the hybridization and extension steps are performed concurrently, at a single temperature.

 In some embodiments, the cyclic polymerase-mediated reaction is performed at a single temperature, and the different processes are accomplished by changing non-thermal properties of the reaction. For example, the denaturation step can be accomplished by
30 incubating the template molecules with a basic solution or other denaturing solution.

 In advantageous embodiments, the percentage of template molecules that are duplicated in the cycle steps is *e.g.* 90%, 70%, 50%, 30%, or less. Such cycles may be as

short as 10, 8, 6, 5, 4.5, 4, 2, 1, 0.5 minutes or less. In certain embodiments, the reaction comprises 2, 5, 10, 15, 20, 30, 40, 50, or more cycles.

Typically, the reactions described herein are repeated until a detectable amount of product is generated. Often, such detectable amounts of product are between about 10 ng and about 100 ng, although larger quantities, *e.g.* 200 ng, 500 ng, 1 mg or more can also, of course, be detected. In terms of concentration, the amount of detectable product can be from about 0.01 pmol, 0.1 pmol, 1 pmol, 10 pmol, or more.

Any of a variety of polymerases can be used in the present invention. For thermocyclic reactions, the polymerases are thermostable polymerases such as Taq, KlenTaq, Stoffel Fragment, Deep Vent, Tth, Pfu, Vent, and UITma, each of which are readily available from commercial sources. Similarly, guidance for the use of each of these enzymes can be readily found in any of a number of protocols found in guides, product literature, and other sources.

For non-thermocyclic reactions, and in certain thermocyclic reactions, the polymerase will often be one of many polymerases commonly used in the field, and commercially available, such as DNA pol 1, Klenow fragment, T7 DNA polymerase, and T4 DNA polymerase. Guidance for the use of such polymerases can readily be found in product literature and in general molecular biology guides.

Those of skill in the art are aware of the variety of nucleotides available for use in the present reaction. Typically, the nucleotides will consist at least in part of deoxynucleotide triphosphates (dNTPs), which are readily commercially available. Parameters for optimal use of dNTPs are also known to those of skill, and are described in the literature. In addition, a large number of nucleotide derivatives are known to those of skill and can be used in the present reaction. Such derivatives include fluorescently labeled nucleotides, allowing the detection of the product including such labeled nucleotides, as described below. Also included in this group are nucleotides that allow the sequencing of nucleic acids including such nucleotides, such as dideoxynucleotides and boronated nuclease-resistant nucleotides, as described below. Other nucleotide analogs include nucleotides with bromo-, iodo-, or other modifying groups, which groups affect numerous properties of resulting nucleic acids including their antigenicity, their replicatability, their melting temperatures, their binding properties, *etc.* In addition, certain nucleotides include reactive side groups, such as

sulfhydryl groups, amino groups, N-hydroxysuccinimidyl groups, that allow the further modification of nucleic acids comprising them.

An oligonucleotide sequence used as a detection probe may be labeled with a detectable moiety. Various labeling moieties are known in the art. Said moiety may, for example, either be a radioactive compound, a detectable enzyme (*e.g.* horse radish peroxidase (HRP)) or any other moiety capable of generating a detectable signal such as a calorimetric, fluorescent, chemiluminescent or electrochemiluminescent signal. Advantageous analysis systems wherein said labels are used are electrochemiluminescence (ECL) based analysis or enzyme linked gel assay (ELGA) based analysis.

In one class of embodiments of this invention, a detectable label is incorporated into a nucleic acid during at least one cycle of the reaction. Spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means can detect such labels. Useful labels in the present invention include fluorescent dyes (*e.g.*, fluorescein isothiocyanate, Texas red, rhodamine, and the like), radiolabels (*e.g.*, ^3H , ^{125}I , ^{35}S , ^{14}C , ^{32}P , *etc.*), enzymes (*e.g.* horseradish peroxidase, alkaline phosphatase *etc.*) colorimetric labels such as colloidal gold or colored glass or plastic (*e.g.* polystyrene, polypropylene, latex, *etc.*) beads. The label is coupled directly or indirectly to a component of the assay according to methods well known in the art. As indicated above, a wide variety of labels are used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions. Non-radioactive labels are often attached by indirect means. Polymerases can also incorporate fluorescent nucleotides during synthesis of nucleic acids.

Reagents allowing the sequencing of reaction products can be utilized herein. For example, chain-terminating nucleotides will often be incorporated into a reaction product during one or more cycles of a reaction. Commercial kits containing the reagents most typically used for these methods of DNA sequencing are available and widely used. PCR exonuclease digestion methods for DNA sequencing can also be used.

Typically, the amplification sequence is serially diluted and then quantitatively amplified via the DNA Tag polymerase using a suitable PCR amplification technique. In PCR, annealing of the primers to the amplification sequence is generally carried out at about 37-50°C.; extension of the primer sequence by Taq polymerase in the presence of nucleoside triphosphates is carried out at about 70-75°C.; and the denaturing step to release the extended

primer is carried out at about 90-95°C. In the two temperature PCR technique, the annealing and extension steps may both be carried at about 60-65°C., thus reducing the length of each amplification cycle and resulting in a shorter assay time.

5 Polymerase chain reactions (PCR) are generally carried out in about 25-50 µl samples containing 0.01 to 1.0 ng of template amplification sequence, 10 to 100 pmol of each generic primer, 1.5 units of Tag DNA polymerase (Promega Corp.), 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 15 mM MgCl₂, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1 µg/ml gelatin, and 10 µl/ml Triton X-100 (Saiki, 1988). Reactions are incubated at 94°C. for 1 minute, about 37 to 55°C. for 2 minutes (depending on the identity of the primers), and
10 about 72°C. for about 3 minutes and repeated for about 5-40, cycles. A two temperature PCR technique differs from the above only in carrying out the annealing/extension steps at a single temperature, e.g., about 60-65°C. for about 5 minutes, rather than at two temperatures.

Another embodiment of the present invention is directed to *ob* gene-specific oligonucleotides that can be used to amplify sequences of *ob* gene DNA, and to subsequently
15 determine if amplification has occurred, from DNA extracted from a livestock specimen. A pair of *ob* gene-specific DNA oligonucleotide primers are used to hybridize to *ob* gene genomic DNA that may be present in DNA extracted from a livestock specimen, and to amplify the specific segment of genomic DNA between the two flanking primers using enzymatic synthesis and temperature cycling. Each pair of primers are designed to hybridize
20 only to the *ob* gene DNA to which they have been synthesized to complement; one to each strand of the double-stranded DNA. The region to which the primers have been synthesized to complement is conserved in *ob* gene. Thus, the reaction is specific even in the presence of microgram quantities of heterologous DNA.

A nucleic acid molecule of the present invention, e.g., a nucleic acid molecule having
25 the nucleotide sequence of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7, or a complement thereof, can be isolated using standard molecular biology techniques and the sequence information provided herein. Using all or a portion of the nucleic acid sequence of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7, as a hybridization probe, nucleic acid sequences can be isolated using standard hybridization and cloning techniques.
30 Furthermore, oligonucleotides can be prepared by standard synthetic techniques, e.g., using an automated DNA synthesizer.

In another embodiment, an isolated nucleic acid molecule of the invention comprises a nucleic acid molecule that is a complement of the nucleotide sequence shown in SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7 or a portion of this nucleotide sequence. A nucleic acid molecule that is complementary to the nucleotide sequence shown in SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7, is one that is sufficiently complementary to the nucleotide sequence shown in SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7 that it can bind with few or no mismatches to the nucleotide sequence shown in SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7, thereby forming a stable duplex.

A nucleic acid molecule of the invention may include only a fragment of the nucleic acid sequence of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7. Fragments provided herein are defined as sequences of at least 6 (contiguous) nucleic acids, a length sufficient to allow for specific hybridization of nucleic acids, and are at most some portion less than a full-length sequence. Fragments may be derived from any contiguous portion of a nucleic acid sequence of choice. Derivatives are nucleic acid sequences formed from the native compounds either directly or by modification or partial substitution. Analogs are nucleic acid sequences that have a structure similar to, but not identical to, the native compound but differ from it in respect to certain components or side chains. Analogs may be synthetic or from a different evolutionary origin and may have a similar or opposite metabolic activity compared to wild type.

Derivatives and analogs may be full length or other than full length, if the derivative or analog contains a modified nucleic acid or amino acid, as described below. Derivatives or analogs of the nucleic acids of the invention include, but are not limited to, molecules comprising regions that are substantially homologous to the nucleic acids of the invention, in various embodiments, by at least about 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or even 99% identity (with an advantageous identity of 80-99%) over a nucleic acid sequence of identical size or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art. Derivatives or analogs of the nucleic acids of the invention also include, but are not limited to, molecules comprising regions that are substantially homologous to the nucleic acids of the invention, in various embodiments, by at least about 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or even 99% identity (with an

advantageous identity of 80-99%) under stringent, moderately stringent, or low stringent conditions.

For the purposes of the present invention, sequence identity or homology is determined by comparing the sequences when aligned so as to maximize overlap and identity while minimizing sequence gaps. In particular, sequence identity may be determined using any of a number of mathematical algorithms. A nonlimiting example of a mathematical algorithm used for comparison of two sequences is the algorithm of Karlin & Altschul, Proc. Natl. Acad. Sci. USA 1990;87: 2264-2268, modified as in Karlin & Altschul, Proc. Natl. Acad. Sci. USA 1993;90: 5873-5877.

Another example of a mathematical algorithm used for comparison of sequences is the algorithm of Myers & Miller, CABIOS 1988;4: 11-17. Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used. Yet another useful algorithm for identifying regions of local sequence similarity and alignment is the FASTA algorithm as described in Pearson & Lipman, Proc. Natl. Acad. Sci. USA 1988;85: 2444-2448.

Advantageous for use according to the present invention is the WU-BLAST (Washington University BLAST) version 2.0 software. WU-BLAST version 2.0 executable programs for several UNIX platforms can be downloaded from ftp://blast.wustl.edu/bblast/executables. This program is based on WU-BLAST version 1.4, which in turn is based on the public domain NCBI-BLAST version 1.4 (Altschul & Gish, 1996, Local alignment statistics, Doolittle ed., Methods in Enzymology 266: 460-480; Altschul et al., Journal of Molecular Biology 1990;215: 403-410; Gish & States, 1993;Nature Genetics 3: 266-272; Karlin & Altschul, 1993;Proc. Natl. Acad. Sci. USA 90: 5873-5877; all of which are incorporated by reference herein).

In all search programs in the suite the gapped alignment routines are integral to the database search itself. Gapping can be turned off if desired. The default penalty (Q) for a gap of length one is Q=9 for proteins and BLASTP, and Q=10 for BLASTN, but may be changed to any integer. The default per-residue penalty for extending a gap (R) is R=2 for proteins and BLASTP, and R=10 for BLASTN, but may be changed to any integer. Any combination of values for Q and R can be used in order to align sequences so as to maximize

overlap and identity while minimizing sequence gaps. The default amino acid comparison matrix is BLOSUM62, but other amino acid comparison matrices such as PAM can be utilized.

Alternatively or additionally, the term "homology" or "identity", for instance, with
5 respect to a nucleotide or amino acid sequence, can indicate a quantitative measure of homology between two sequences. The percent sequence homology can be calculated as $(N_{ref} - N_{dif}) * 100 / N_{ref}$, wherein N_{dif} is the total number of non-identical residues in the two sequences when aligned and wherein N_{ref} is the number of residues in one of the sequences. Hence, the DNA sequence AGTCAGTC will have a sequence identity of 75%
10 with the sequence AATCAATC ($N_{ref} = 8$; $N_{dif} = 2$).

Alternatively or additionally, "homology" or "identity" with respect to sequences can refer to the number of positions with identical nucleotides or amino acids divided by the number of nucleotides or amino acids in the shorter of the two sequences wherein alignment of the two sequences can be determined in accordance with the Wilbur and Lipman algorithm
15 (Wilbur & Lipman, Proc Natl Acad Sci USA 1983;80:726, incorporated herein by reference), for instance, using a window size of 20 nucleotides, a word length of 4 nucleotides, and a gap penalty of 4, and computer-assisted analysis and interpretation of the sequence data including alignment can be conveniently performed using commercially available programs (e.g., Intelligenetics™ Suite, Intelligenetics Inc. CA). When RNA sequences are said to be
20 similar, or have a degree of sequence identity or homology with DNA sequences, thymidine (T) in the DNA sequence is considered equal to uracil (U) in the RNA sequence. Thus, RNA sequences are within the scope of the invention and can be derived from DNA sequences, by thymidine (T) in the DNA sequence being considered equal to uracil (U) in RNA sequences.

And, without undue experimentation, the skilled artisan can consult with many other
25 programs or references for determining percent homology.

The nucleotide sequence of probes and primers typically comprises a substantially purified oligonucleotide. The oligonucleotide typically comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least about 6, 9, 12, 16, 24, or more consecutive sense strand nucleotide sequence of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6
30 or SEQ ID NO:7, or an anti-sense strand nucleotide sequence of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7, or of a naturally occurring mutant of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7.

In various embodiments, the probe further comprises a label group attached thereto. Such probes can be used as a part of a diagnostic test kit for assessing the presence of homozygous mutant alleles of the *ob* gene (*ob⁻/ob⁻* or TT animals), heterozygous mutant alleles of the *ob* gene (*ob⁻/ob⁺* or CT animals) and wild-type alleles of the *ob* gene (*ob⁺/ob⁺* or CC animals).

The invention further encompasses nucleic acid molecules that differ from the nucleotide sequences shown in SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7, due to the degeneracy of the genetic code.

In addition to the nucleotide sequence shown in SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7, it will be appreciated by those skilled in the art that DNA sequence polymorphisms in the *ob* gene DNA may exist within a population. Such natural allelic variations can typically result in about 1-5% variance in the nucleotide sequence of the gene. Any and all such nucleotide variations are intended to be within the scope of the invention.

Moreover, nucleic acid molecules that differ from the sequence of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7, are intended to be within the scope of the invention. Nucleic acid molecules corresponding to natural allelic variants and homologues of the DNAs of the invention can be isolated based on their homology to the nucleic acids disclosed herein using standard hybridization techniques under stringent hybridization conditions. Advantageously, such variations will differ from the sequence of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7, by only one nucleotide.

Accordingly, in another embodiment, an isolated nucleic acid molecule of the invention is at least 6 nucleotides in length and hybridizes under stringent conditions to the nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7.

Homologs (*i.e.*, nucleic acids derived from other species) or other related sequences (*e.g.*, paralogs) can be obtained under conditions of standard or stringent hybridization conditions with all or a portion of the particular sequence as a probe using methods well known in the art for nucleic acid hybridization and cloning.

In another embodiment, a nucleic acid sequence that is hybridizable to the nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:4, SEQ ID NO:5, SEQ ID

NO:6 or SEQ ID NO:7, or fragments, analogs or derivatives thereof, under conditions of standard or stringent hybridization conditions is provided.

In addition to naturally-occurring allelic variants of the nucleotide sequence, the skilled artisan will further appreciate that changes can be introduced by mutation into the nucleotide sequence of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7.

In one embodiment, the isolated nucleic acid molecule comprises a nucleotide sequence at least about 75% homologous to the nucleotide sequence of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7. Advantageously, the nucleic acid is at least about 80% homologous to the nucleotide sequence of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7, more advantageously at least about 90%, 95%, 96%, 97%, 98%, and most advantageously at least about 99% homologous to the nucleotide sequence of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7.

As already indicated above, and will be presented in the experimental part of the description, both the sensitivity and reliability of polymorphism detection is greatly improved using the oligonucleotides according to the present invention when compared to known methods used in this art.

It is understood that oligonucleotides consisting of the sequences of the present invention may contain minor deletions, additions and/or substitutions of nucleic acid bases, to the extent that such alterations do not negatively affect the yield or product obtained to a significant degree.

Test kits for assessing the presence of homozygous mutant alleles of the *ob* gene (*ob⁻/ob⁻* or TT animals), heterozygous mutant alleles of the *ob* gene (*ob⁻/ob⁺* or CT animals) and wild-type alleles of the *ob* gene (*ob⁺/ob⁺* or CC animals) are also part of the present invention. A test kit according to the invention may comprise a pair of oligonucleotides according to the invention and a probe comprising an oligonucleotide according to the invention. Such a test kit may additionally comprise suitable amplification reagents such as DNA and or RNA polymerases and mononucleotides. Test kits that can be used with the method according to the invention may comprise the oligonucleotides according to the invention for the amplification and subsequent assessment of for the presence of homozygous mutant alleles of the *ob* gene (*ob⁻/ob⁻* or TT animals), heterozygous mutant alleles of the *ob* gene (*ob⁻/ob⁺* or CT animals) and wild-type alleles of the *ob* gene (*ob⁺/ob⁺* or CC animals). An advantageous embodiment for the test kit comprises the oligonucleotides: SEQ ID NO:4

and SEQ ID NO:5 as primer pairs for the amplification, and oligonucleotides SEQ ID NO:6 or SEQ ID NO:7, for use with SEQ ID NO:4 and SEQ ID NO:5, provided with a detectable label, as probes.

5 A diagnostic test kit for detection of *ob* gene according to the compositions and methods of the present invention may include, in separate packaging, a lysing buffer for lysing cells contained in the specimen; at least one oligonucleotide primer pair (SEQ ID NO:4 and SEQ ID NO:5); enzyme amplification reaction components such as dNTPs, reaction buffer, and/or amplifying enzyme; and at least one oligonucleotide probe labeled with a detectable moiety (SEQ ID NO:6 or SEQ ID NO:7), or various combinations thereof.

10 The present invention further provides nucleic acid detection kits, including arrays or microarrays of nucleic acid molecules that are based on one or more of the sequences provided in SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:7. As used herein "Arrays" or "Microarrays" refers to an array of distinct polynucleotides or oligonucleotides synthesized on a solid or flexible support, such as paper, nylon or other type of membrane,
15 filter, chip, glass slide, or any other suitable solid support. In one embodiment, the microarray is prepared and used according to the methods and devices described in U.S. Pat. Nos. 5,446,603; 5,545,531; 5,807,522; 5,837,832; 5,874,219; 6,114,122; 6,238,910; 6,365,418; 6,410,229; 6,420,114; 6,432,696; 6,475,808 and 6,489,159 and PCT Publication No. WO 01/45843 A2, the disclosures of which are incorporated by reference in their
20 entireties.

Although the above methods are described in terms of the use of a single probe and a single set of primers, the methods are not so limited. One or more additional probes and/or primers can be used, if desired. Additional enzymes, constructed probes and primers can be determined through routine experimentation.

25 The reagents suitable for applying the methods of the invention may be packaged into convenient kits. The kits provide the necessary materials, packaged into suitable containers. Advantageously, the containers are also supports useful in performing the assay. At a minimum, the kit contains a reagent that identifies a polymorphism in the livestock *ob* gene that is associated with an increased weight gain. Advantageously, the reagent is a probe
30 and/or PCR set (a set of primers, DNA polymerase and 4 nucleoside triphosphates) that hybridize with the livestock *ob* gene or a fragment thereof.

Advantageously, both the probe (or PCR set) and a restriction enzyme that cleaves the livestock *ob* gene in at least one place are included in the kit. In a particularly advantageous embodiment of the invention, the probe comprises the human *ob* gene, the livestock *ob* gene, or a gene fragment that has been labeled with a detectable entity. Advantageously, the kit
5 further comprises additional means, such as reagents, for detecting or measuring the detectable entity or providing a control. Other reagents used for hybridization, prehybridization, DNA extraction, *etc.* may also be included, if desired.

The methods and materials of the invention may also be used more generally to evaluate livestock DNA, genetically type individual livestock, and detect genetic differences
10 in livestock. In particular, a sample of livestock genomic DNA may be evaluated by reference to one or more controls to determine if a polymorphism in the *ob* gene is present. Any method for determining genotype can be used for determining the *ob* genotype in the present invention. Such methods include, but are not limited to, amplicon sequencing, DNA sequencing, fluorescence spectroscopy, FRET-based hybridization analysis, high throughput
15 screening, mass spectroscopy, microsatellite analysis, nucleic acid hybridization, polymerase chain reaction (PCR), RFLP analysis and size chromatography (e.g., capillary or gel chromatography), all of which are well known to one of skill in the art. In particular, methods for determining nucleotide polymorphisms, particularly single nucleotide polymorphisms, are described in U.S. Patent Nos. 6,514,700; 6,503,710; 6,468,742;
20 6,448,407; 6,410,231; 6,383,756; 6,358,679; 6,322,980; 6,316,230; and 6,287,766 and reviewed by Chen and Sullivan, *Pharmacogenomics J* 2003;3(2):77-96, the disclosures of which are incorporated by reference in their entireties.

Advantageously, FRET analysis is performed with respect to the livestock *ob* gene, and the results are compared with a control. The control is the result of a FRET analysis of
25 the livestock *ob* gene of a different livestock where the polymorphism of the livestock *ob* gene is known. Similarly, the estrogen receptor genotype of a livestock may be determined by obtaining a sample of its genomic DNA, conducting FRET analysis of the *ob* gene in the DNA, and comparing the results with a control. Again, the control is the result of FRET analysis of the *ob* gene of a different livestock. The results genetically type the livestock by
30 specifying the polymorphism in its *ob* genes. Finally, genetic differences among livestock can be detected by obtaining samples of the genomic DNA from at least two livestock,

identifying the presence or absence of a polymorphism in the *ob* gene, and comparing the results.

These assays are useful for identifying the genetic markers relating to weight gain, as discussed above, for identifying other polymorphisms in the *ob* gene that may be correlated with other characteristics, and for the general scientific analysis of livestock genotypes and phenotypes.

The genetic markers, methods, and kits of the invention are also useful in a breeding program to improve feed conversion efficiency in a breed, line, or population of livestock. Continuous selection and breeding of livestock that are at least heterozygous and advantageously homozygous for a polymorphism associated with increased feed conversion efficiency would lead to a breed, line, or population having higher numbers of offspring in each litter of the females of this breed or line. Thus, the markers can be used as selection tools.

It is to be understood that the application of the teachings of the present invention to a specific problem or environment will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. The examples of the products and processes of the present invention appear in the following examples.

Further, the invention provides a method of using oligonucleotide primers (SEQ ID No.2 & SEQ ID No 3) based on this DNA sequence in a polymerase chain reaction (PCR) assay to distinguish livestock animals homozygous for mutant alleles of the *ob* gene (*ob⁻/ob⁻* or TT animals), which alleles encode an altered leptin, from livestock animals heterozygous for mutant alleles of the *ob* gene (*ob⁻/ob⁺* or CT animals) and livestock animals homozygous for wild-type alleles of the *ob* gene (*ob⁺/ob⁺* or CC animals).

In another embodiment, the invention provides a method of using primers having SEQ ID No.2 & SEQ ID No 3 based on this DNA sequence in a polymerase chain reaction (PCR) assay to distinguish livestock animals homozygous for mutant alleles of the *ob* gene (*ob⁻/ob⁻* or TT animals), which alleles encode an altered leptin, from livestock animals heterozygous for mutant alleles of the *ob* gene (*ob⁻/ob⁺* or CT animals) and livestock animals homozygous for wild-type alleles of the *ob* gene (*ob⁺/ob⁺* or CC animals), wherein detection of the PCR amplified fragment is by detection of a radioactively labeled nucleotide that is incorporated into the PCR amplified product.

In yet another embodiment, a non-radioactively labeled nucleotide is incorporated into the PCR amplified product and detection is by colorimetry, chemiluminescence, or measurement of fluorescence.

5 In another embodiment, the method of detection is based on the use of fluorescently labeled nucleotides in Fluorescence Resonance Energy Transfer (FRET) based detection systems including Taqman, Molecular Beacon, *etc.*, which are familiar to those conversant with prior art.

10 The oligonucleotides in the present invention can be produced by a conventional production process for general oligonucleotides. It can be produced, for example, by a chemical synthesis process or by a microbial process which makes use of a plasmid vector, a phage vector or the like (Tetrahedron Letters, 22, 1859-1862, 1981; Nucleic Acids Research, 14, 6227-6245, 1986). Further, it is suitable to use a nucleic acid synthesizer currently available on the market.

15 To label an oligonucleotide with the fluorescent dye, one of conventionally-known labeling methods can be used (Nature Biotechnology, 14, 303-308, 1996; Applied and Environmental Microbiology, 63, 1143-1147, 1997; Nucleic Acids Research, 24, 4532-4535, 1996). Reversed phase chromatography or the like used to provide a nucleic acid probe for use in the present invention can purify the synthesized oligonucleotide, which is labeled with the fluorescent dye.

20 The nucleic acid probe according to the present invention can be prepared as described above. An advantageous probe form is one labeled with a fluorescent dye at the 3' or 5' end and containing G or C as the base at the labeled end. If the 5' end is labeled and the 3' end is not labeled, the OH group on the C atom at the 3'-position of the 3' end ribose or deoxyribose may be modified with a phosphate group or the like although no limitation is imposed in this respect.

25 Inclusion of the nucleic acid probe according to the present invention in a kit for analyzing or determining polymorphism and/or mutation of a target nucleic acid or gene, therefore, makes it possible to suitably use the kit as a kit for the analysis or determination of the polymorphism and/or mutation of the target nucleic acid or gene.

30 The probe according to the present invention may be immobilized on a surface of a solid (support layer), for example, on a surface of a slide glass. In this case, the probe may advantageously be immobilized on the end not labeled with the fluorescent dye. The probe of

this form is now called a "DNA chip". These DNA chips can be used for monitoring gene expressions, determining base sequences, analyzing mutations or analyzing polymorphisms such as single nucleotide polymorphism (SNP). They can also be used as devices (chips) for determining nucleic acids.

5 In one aspect, during the hybridization of the nucleic acid target with the probes, stringent conditions may be utilized, advantageously along with other stringency affecting conditions, to aid in the hybridization. Detection by differential disruption is particularly advantageous to reduce or eliminate slippage hybridization among probes and target, and to promote more effective hybridization. In yet another aspect, stringency conditions may be
10 varied during the hybridization complex stability determination so as to more accurately or quickly determine whether a SNP is present in the target sequence.

 Thus, the present invention provides for a method of determining a polymorphism comprising (a) obtaining a nucleic acid sample; (b) hybridizing the nucleic acid sample with a probe, and (c) disrupting the hybridization to determine the level of disruption energies
15 required wherein the sensor probe has a different disruption energy if there is a mutation in the homology between the original nucleic acid sequence and sensor probe for hybridization. In one example, there is a lower disruption energy, e.g., melting temperature, for an allele that harbors the mutation site, and a higher required energy for an allele with no mutation since the homology is 100% and therefore requires more energy to cause the hybridized
20 target to dissociate.

 Optionally, in step (b) a second ("anchor") probe used. Generally, the anchor probe is not specific to either t or c allele, but hybridizes regardless whether there is a c or t allele. The anchor probe does not affect the disruption energy required to disassociate the hybridization complex but, instead, contains a complementary label for using with the first
25 ("sensor") probe.

 Hybridization stability may be influenced by numerous factors, including thermoregulation, chemical regulation, as well as electronic stringency control, either alone or in combination with the other listed factors. Through the use of stringency conditions, in either or both of the target hybridization step or the sensor oligonucleotide stringency step,
30 rapid completion of the process may be achieved. This is desirable to achieve properly indexed hybridization of the target DNA to attain the maximum number of molecules at a test site with an accurate hybridization complex. By way of example, with the use of stringency,

the initial hybridization step may be completed in ten minutes or less, more advantageously five minutes or less, and most advantageously two minutes or less. Overall, the analytical process may be completed in less than half an hour.

As to detection of the hybridization complex, it is advantageous that the complex is
5 labeled. Typically, in the step of determining hybridization of probe to target, there is a detection of the amount of labeled hybridization complex at the test site or a portion thereof. Any mode or modality of detection consistent with the purpose and functionality of the invention may be utilized, such as optical imaging, electronic imaging, use of charge-coupled devices or other methods of quantification. Labeling may be of the target, capture, or sensor.
10 Various labeling may be by fluorescent labeling, colormetric labeling or chemiluminescent labeling. In yet another implementation, detection may be via energy transfer between molecules in the hybridization complex. In yet another aspect, the detection may be via fluorescence perturbation analysis. In another aspect the detection may be via conductivity differences between concordant and discordant sites.

15 In yet another aspect, detection can be carried out using mass spectrometry. In such method, no fluorescent label is necessary. Rather detection is obtained by extremely high levels of mass resolution achieved by direct measurement, for example, by time of flight or by electron spray ionization (ESI). Where mass spectrometry is contemplated, sensor probes having a nucleic acid sequence of 50 bases or less are advantageous.

20 In one mode, the hybridization complex is labeled and the step of determining amount of hybridization includes detecting the amounts of labeled hybridization complex at the test sites. The detection device and method may include, but is not limited to, optical imaging, electronic imaging, imaging with a CCD camera, integrated optical imaging, and mass spectrometry. Further, the detection, either labeled or unlabeled, is quantified, which may
25 include statistical analysis. The labeled portion of the complex may be the target, the stabilizer, the sensor or the hybridization complex in toto. Labeling may be by fluorescent labeling selected from the group of, but not limited to, Cy3, Cy5, Bodipy Texas Red, Bodipy Far Red, Lucifer Yellow, Bodipy 630/650-X, Bodipy R6G-X and 5-CR 6G. Labeling may further be accomplished by colormetric labeling, bioluminescent labeling and/or
30 chemiluminescent labeling. Labeling further may include energy transfer between molecules in the hybridization complex by perturbation analysis, quenching, electron transport between donor and acceptor molecules, the latter of which may be facilitated by double stranded

match hybridization complexes. Optionally, if the hybridization complex is unlabeled, detection may be accomplished by measurement of conductance differential between double stranded and non-double stranded DNA. Further, direct detection may be achieved by porous silicon-based optical interferometry or by mass spectrometry.

5 The label may be amplified, and may include for example branched or dendritic DNA. If the target DNA is purified, it may be unamplified or amplified. Further, if the purified target is amplified and the amplification is an exponential method, it may be, for example, PCR amplified DNA or strand displacement amplification (SDA) amplified DNA. Linear methods of DNA amplification such as rolling circle or transcriptional runoff may also
10 be used.

By way of example, following incubation of the sensor probes, discrimination is achieved by subjecting the complex to destabilizing conditions, e.g., heating the complex to about 4°C below melting temperature of the perfectly matched sensor/amplicon in a low salt buffer (e.g., 50 mM NaPO₄). For FRET, imaging is then performed using two different
15 lasers, one corresponding to the fluorophore on the wild-type sensor and one to the fluorophore on the mutant sensor. From these signal intensities, backgrounds are subtracted and specific activities are taken into account. A determination of wild type and mutant signals is achieved from which the allelic compositions of the amplicon products are determined.

20 In one embodiment, the method comprises (a) contacting the target nucleic acid of interest with at least one sensor oligonucleotide, wherein the sensor oligonucleotide comprises a sequence complementary to at least a portion of the target nucleic acid of interest, wherein the sensor oligonucleotide hybridizes to the target nucleic acid at a position suspected of containing the *ob* gene polymorphism and (b) subjecting the captured target
25 nucleic acid and hybridized sensor probe oligonucleotide to destabilizing conditions, wherein the destabilizing conditions are sufficient to cause the sensor oligonucleotide to dissociate under differing conditions depending upon the presence of the cc, ct or tt polymorphisms in the *ob* gene.

30 In another embodiment, the method further comprises (c) detecting the hybridization of the sensor oligonucleotide to the target nucleic acid under the varying destabilizing conditions, whereby the presence of the specific sequence in the target nucleic acid is determined.

In yet another embodiment, the method further comprises a preparatory step of amplifying one or more target nucleic acid sequences from the nucleic acids of a sample, wherein the amplicons become the target nucleic acids.

5 In one embodiment, the amplification step produces single stranded amplicons, which are then utilized as the single stranded target nucleic acids. In another embodiment, the amplification step produces double stranded amplicons, further comprising a step of subjecting the amplicons to denaturing conditions to form single stranded target nucleic acids.

10 In an alternate embodiment, the amplification step is by an amplification method selected from the group consisting of polymerase chain reaction (PCR), strand displacement amplification (SDA), nucleic acid sequence-based amplification (NASBA), rolling circle amplification, T7 mediated amplification, T3 mediated amplification, and SP6 mediated amplification.

15 In one embodiment, the method comprising a step of subjecting the target nucleic acids of the sample to denaturing conditions to form single stranded target nucleic acids.

In another embodiment, the detection of the hybridization of the sensor oligonucleotide is by the detection of a labeling moiety on the sensor oligonucleotide selected from the group consisting of fluorescent moieties, bioluminescent moieties, chemiluminescent moieties, and colorigenic moieties. Advantageously, the labeling moiety
20 is a fluorescent moiety selected from the group consisting of fluorescein derivatives, BODIPYL dyes, rhodamine derivatives, Lucifer Yellow derivatives, and cyanine (Cy) dyes.

In an alternate embodiment, the destabilizing conditions are created by methods selected from the group consisting of making temperature adjustments, making ionic strength adjustments, making adjustments in pH, and combinations thereof.

25 In one embodiment, the method comprises (a) contacting a single stranded target nucleic acid of interest with (i) a first sensor oligonucleotide, wherein the first sensor oligonucleotide comprises a sequence complementary to at least a portion of the target nucleic acid of interest; (ii) further contacting the target nucleic acid with at least a second sensor oligonucleotide, wherein the second sensor oligonucleotide comprises a sequence
30 complementary to at least a portion of the target nucleic acid of interest; (b) subjecting the target nucleic acid and hybridized sensor oligonucleotides to destabilizing conditions, wherein the destabilizing conditions are sufficient to cause the first and/or second sensor

oligonucleotide to dissociate under different destabilizing conditions; and (c) detecting the hybridization of the first and second sensor oligonucleotide to the target nucleic acid, whereby the presence of the polymorphism in the target nucleic acid is determined. Advantageously, the first and second sensor oligonucleotides are differently labeled with first
5 and second labeling moieties.

In detecting a polymorphisms by differential melting temperature, the region surrounding the SNP is amplified by PCR or other amplification method. In another embodiment, a detectable label is incorporated into the system, either by use of a labeled primer, a labeled nucleotide, a labeled ribonucleotide, a labeled, modified nucleotide or a
10 labeled, modified ribonucleotide. Alternatively, a label may be incorporated after selective hybridization has occurred, *i.e.* after the temperature has been raised to a degree whereby at least one of the fragments dissociates from the oligonucleotide probe.

The cleavage products are hybridized to oligonucleotide probes designed to maximize the difference in hybridization signal obtained from the two different alleles. For optimal
15 detection of single-base pair mismatches, an about a 1°C to about 10°C difference in melting temperature is advantageous. When the temperature is raised above the melting temperature of a fragment-oligonucleotide duplex corresponding to one of the alleles, that allele will disassociate. The remaining fragment-oligonucleotide duplexes can then be analyzed for the incorporated label that identified the polymorphism.

20 The present invention provides methods for identifying the presence of one or more SNP allele in a diploid DNA sample. The detection occurs when there is a loss of florescence emitted by the sensor probe. The sensor probe acquires energy from the anchor probe once conditions are adequate for hybridization between the target (genomic) DNA and the anchor and sensor probe. Once hybridization occurs, the anchor probe transfers its florescence
25 energy to the sensor probe, which only will emit a specific wavelength after it has acquired the energy from the anchor probe. Detection of the SNP occurs as the temperature is raised at a predetermined rate, and a reading is acquired from the florescent light emitted. If there is a presence of the mutation (SNP) the sensor probe will dissociate sooner, or at a lower temperature, since the homology between the genomic DNA and the sensor probe will be less
30 than that of genomic DNA that does not harbor the SNP. The melt occurs lower in the case of the DNA with the SNP since the stability is compromised slightly. This occurs, obviously, on both chromosomes at the same time, thus yielding either a reading of two identical melting

temperatures, or a reading of two different melting temperatures, being the heterozygote. The individuals that harbor two copies of the SNP, dubbed "tt" melt at approximately 54 °C, and the individuals containing only wild type DNA (no SNP present), dubbed "cc", melt at approximately 63 °C.

5 In one embodiment, the leptin (*ob*) mutation is genotyped as "tt" if the sample melts only at a low temperature (generally, at about 54°C), as "ct" if the sample melts at both a high and a low temperature (generally, about 54°C and about 63°C), and "cc" if it melts at only the high temperature (generally, about 63°C). The melting temperatures are generally within about 4°C, advantageously within about 2°C.

10 In one embodiment of the invention, the oligonucleotide probes used in the above assays can be immobilized on a solid support such as, without limitation, microchips, microbeads, glass slides or any other such matrix, all of which are within the scope of this invention.

15 Using an assay of this type, a fluorescent labeled probe anneals to the denatured single strand. When the probe hybridizes to any specific target sequence produced as a result of the amplification reaction, the reactive molecule absorbs emission energy from labeled nucleotides or donates energy to the labeled nucleotides by means of FET or FRET, thus changing the signal from the fluorescent nucleotides. Advantageously, the receptor probe takes the energy emitted from the donor probe and emits energy at a different wavelength, 20 which is then measured. This new wavelength emission may be detected and this indicates binding of the probe. Alternatively, the reactive molecule is able to absorb fluorescence from the labeled nucleotides and so the fluorescence from these is reduced. This reduction may be detected and this indicates binding of the probe.

25 Most advantageously, the reactive molecule is an acceptor molecule which it emits fluorescence at a characteristic wavelength. In this case, increase in fluorescence from the acceptor molecule, which is of a different wavelength to that of the labeled nucleotide, will also indicate binding of the probe.

30 The presence of the labeled amplification product can be detected by monitoring fluorescence from the acceptor molecule on the probe, which specifically binds only the target sequence. In this case, signal from the amplification product can be distinguished from background signal of the fluorescent label and also from any non-specific amplification product.

An assay of this nature can be carried out using inexpensive reagents. Single labeled probes are more economical to those that include both acceptor and donor molecules.

As used herein, the expression "set of nucleotides" refers to a group of nucleotides that are sufficient to form nucleic acids such as DNA and RNA. Thus these comprise
5 adenosine, cytosine, guanine and thymine or uracil. One or more of these is fluorescently labeled.

Amplification is suitably effected using known amplification reactions such as the polymerase chain reaction (PCR) or the ligase chain reaction (LCR), strand displacement assay (SDA) or NASBA, but advantageously PCR.

10 In some embodiments, the fluorescence of both the nucleotide and the acceptor molecule are monitored and the relationship between the emissions calculated.

Suitable reactive molecules (such as acceptor molecules) are rhodamine dyes or other dyes such as Cy5. These may be attached to the probe in a conventional manner. The position of the reactive molecule along the probe is immaterial although in general, they will
15 be positioned at an end region of the probe.

In order for FET, such as FRET, to occur between the reactive molecule and fluorescent emission of the nucleotides, the fluorescent emission of the element (reactive molecule or labeled nucleotide) which acts as the donor must be of a shorter wavelength than the element acceptor. Suitable combinations are SYBRGold and rhodamine; SYBRGreen I
20 and rhodamine; SYBRGold and Cy5; SYBRGreen I and Cy5; and fluorescein and ethidium bromide

Advantageously, the molecules used as donor and/or acceptor produce sharp peaks, and there is little or no overlap in the wavelengths of the emission. Under these circumstances, it may not be necessary to resolve the "strand specific peak" from the signal
25 produced by amplification product. A simple measurement of the strand specific signal alone (*i.e.* that provided by the reactive molecule) will provide information regarding the extent of the FET or FRET caused by the target reaction. The ethidium bromide/fluorescein combination may fulfill this requirement. In that case, the strand specific reaction will be quantifiable by the reduction in fluorescence at 640 nm, suitably expressed as 1/Fluorescence.

30 However, where there is a spectral overlap in the fluorescent signals from the donor and acceptor molecules, this can be accounted for in the results, for example by determining

empirically the relationship between the spectra and using this relationship to normalize the signals from the two signals.

5 In one method of the invention, the sample may be subjected to conditions under which the probe hybridizes to the samples during or after the amplification reaction has been completed. The process allows the detection to be effected in a homogenous manner, in that the amplification and monitoring can be carried out in a single container with all reagents added initially. No subsequent reagent addition steps are required. Neither is there any need to effect the method in the presence of solid supports (although this is an option as discussed further hereinafter).

10 For example, where the probe is present throughout the amplification reaction, the fluorescent signal may allow the progress of the amplification reaction to be monitored. This may provide a means for quantitating the amount of target sequence present in the sample.

15 During each cycle of the amplification reaction, amplicon strands containing the target sequence bind to probe and thereby generate an acceptor signal. As the amount of amplicon in the sample increases, so the acceptor signal will increase. By plotting the rate of increase over cycles, the start point of the increase can be determined.

20 The probe may comprise a nucleic acid molecule such as DNA or RNA, which will hybridize to the target nucleic acid sequence when the latter is in single stranded form. In this instance, step (b) will involve the use of conditions which render the target nucleic acid single stranded. Alternatively, the probe may comprise a molecule such as a peptide nucleic acid that specifically binds the target sequence in double stranded form.

In particular, the amplification reaction used will involve a step of subjecting the sample to conditions under which any of the target nucleic acid sequence present in the sample becomes single stranded, such as PCR or LCR.

25 It is possible then for the probe to hybridize during the course of the amplification reaction provided appropriate hybridization conditions are encountered.

30 In an advantageous embodiment, the probe may be designed such that these conditions are met during each cycle of the amplification reaction. Thus at some point during each cycle of the amplification reaction, the probe will hybridize to the target sequence, and generate a signal as a result of the FET or FRET. As the amplification proceeds, the probe will be separated or melted from the target sequence and so the signal generated by the reactive molecule will either reduce or increase depending upon whether it comprises the

donor or acceptor molecule. For instance, where it is an acceptor, in each cycle of the amplification, a fluorescence peak from the reactive molecule is generated. The intensity of the peak will increase as the amplification proceeds because more target sequence becomes available for binding to the probe.

5 By monitoring the fluorescence of the reactive molecule from the sample during each cycle, the progress of the amplification reaction can be monitored in various ways. For example, the data provided by melting peaks could be analyzed, for example by calculating the area under the melting peaks and this data plotted against the number of cycles.

10 The probe may either be free in solution or immobilized on a solid support, for example to the surface of a bead such as a magnetic bead, useful in separating products, or the surface of a detector device, such as the waveguide of a surface plasma resonance detector. The selection will depend upon the nature of the particular assay being looked at and the particular detection means being employed.

15 An increase in fluorescence of the acceptor molecule in the course of or at the end of the amplification reaction is indicative of an increase in the amount of the target sequence present, suggestive of the fact that the amplification reaction has proceeded and therefore the target sequence was in fact present in the sample.

20 Thus, one embodiment of the invention comprises a method for detecting nucleic acid amplification comprising: performing nucleic acid amplification on a target polynucleotide in the presence of (a) a nucleic acid polymerase (b) at least one primer capable of hybridizing to the target polynucleotide, (c) a set of nucleotides, at least one of which is fluorescently labeled and (d) an oligonucleotide probe which is capable of binding to the target polynucleotide sequence and which contains a reactive molecule which is capable of absorbing fluorescence from or donating fluorescence to the labeled nucleotide; and
25 monitoring changes in fluorescence during the amplification reaction. Suitably, the reactive molecule is an acceptor molecule that can absorb energy from the labeled nucleotide.

30 The amplification is suitably carried out using a pair of primers which are designed such that only the target nucleotide sequence within a DNA strand is amplified as is well understood in the art. The nucleic acid polymerase is suitably a thermostable polymerase such as Taq polymerase.

Suitable conditions under which the amplification reaction can be carried out are well known in the art. The optimum conditions may be variable in each case depending upon the

particular amplicon involved, the nature of the primers used and the enzymes employed. The optimum conditions may be determined in each case by the skilled person. Typical denaturation temperatures are of the order of 95°C, typical annealing temperatures are of the order of 55°C and extension temperatures are of the order of 72°C

5 Alternatively or additionally, the method of the invention can be used in hybridization assays for determining characteristics of a sequence. Thus in a further aspect, the invention provides a method for determining a characteristic of a sequence, the method comprising (a) amplifying the sequence using a set of nucleotides, at least one of which is fluorescently labeled, (b) contacting amplification product with a probe under conditions in which the
10 probe will hybridize to the target sequence, the probe comprising a reactive molecule which is able to absorb fluorescence from or donate fluorescent energy to the fluorescent labeled nucleotide and (c) monitoring fluorescence of the sample and determining a particular reaction condition, characteristic of the sequence, at which fluorescence changes as a result of the hybridization of the probe to the sample or destabilization of the duplex formed between
15 the probe and the target nucleic acid sequence.

Suitable reaction conditions include temperature, electrochemical, or the response to the presence of particular enzymes or chemicals. By monitoring changes in fluorescence as these properties are varied, information characteristic of the precise nature of the sequence can be achieved. For example, in the case of temperature, the temperature at which the probe
20 separates from the sequences in the sample as a result of heating can be determined.

Another way to produce a FRET signal that discriminates between the two variant alleles is to incorporate a nucleotide with a dye that interacts with the dye on the primer. The key to achieving differential FRET is that the dye modified nucleotide must first occur (after the 3' end of the primer) beyond the polymorphic site so that, after cleavage, the nucleotide
25 dye of one allele (cleaved) will no longer be in within the requisite resonance producing distance of the primer dye while, in the other (uncleaved) allele, the proper distance will be maintained and FRET will occur.

In the present invention, the above-described nucleic acid probe is added to a measurement system and is caused to hybridize to a target nucleic acid. This hybridization
30 can be by conventionally known methods. As conditions for hybridization, the salt concentration may range from 0 to 2 molar concentration, advantageously from 0.1 to 1.0 molar concentration, and the pH may range from 6 to 8, advantageously from 6.5 to 7.5.

The reaction temperature may advantageously be in a range of the T_m value of the hybrid complex, which is to be formed by hybridization of the nucleic acid probe to the specific site of the target nucleic acid, $\pm 10^\circ\text{C}$. This temperature range can prevent non-specific hybridization. Reaction temperature lower than $T_m - 10^\circ\text{C}$ allows non-specific hybridization, while a reaction temperature higher than $T_m + 10^\circ\text{C}$ allows no hybridization. Incidentally, a T_m value can be determined in a similar manner as in an experiment that is needed to design the nucleic acid probe for use in the present invention. Described specifically, an oligonucleotide which is to be hybridized with the nucleic acid probe and has a complementary base sequence to the nucleic acid probe is chemically synthesized by the above-described nucleic acid synthesizer or the like, and the T_m value of a hybrid complex between the oligonucleotide and the nucleic acid probe is then measured by a conventional method.

The reaction time may range from 1 second to 180 minutes, advantageously from 5 seconds to 90 minutes. If the reaction time is shorter than 1 second, a substantial portion of the nucleic acid probe according to the present invention remains unreacted in the hybridization. On the other hand, no particular advantage can be brought about even if the reaction time is set excessively long. The reaction time varies considerably depending on the kind of the nucleic acid, namely, the length or base sequence of the nucleic acid.

In the present invention, the nucleic acid probe is hybridized to the target nucleic acid as described above. The intensity of fluorescence emitted from the fluorescent dye is measured both before and after the hybridization, and a decrease in fluorescence intensity after the hybridization is then calculated. As the decrease is proportional to the concentration of the target nucleic acid, the concentration of the target nucleic acid can be determined.

In certain embodiments of the present invention, the detection of polymorphic sites in a target polynucleotide may be facilitated through the use of nucleic acid amplification methods. Such methods may be used to specifically increase the concentration of the target polynucleotide (*i.e.*, sequences that span the polymorphic site, or include that site and sequences located either distal or proximal to it). Such amplified molecules can be readily detected by gel electrophoresis, or other means.

The most advantageous method of achieving such amplification employs PCR (see *e.g.*, U.S. Pat. Nos. 4,965,188; 5,066,584; 5,338,671; 5,348,853; 5,364,790; 5,374,553; 5,403,707; 5,405,774; 5,418,149; 5,451,512; 5,470,724; 5,487,993; 5,523,225; 5,527,510;

5,567,583; 5,567,809; 5,587,287; 5,597,910; 5,602,011; 5,622,820; 5,658,764; 5,674,679; 5,674,738; 5,681,741; 5,702,901; 5,710,381; 5,733,751; 5,741,640; 5,741,676; 5,753,467; 5,756,285; 5,776,686; 5,811,295; 5,817,797; 5,827,657; 5,869,249; 5,935,522; 6,001,645; 6,015,534; 6,015,666; 6,033,854; 6,043,028; 6,077,664; 6,090,553; 6,168,918; 6,174,668; 5 6,174,670; 6,200,747; 6,225,093; 6,232,079; 6,261,431; 6,287,769; 6,306,593; 6,440,668; 6,468,743; 6,485,909; 6,511,805; 6,544,782; 6,566,067; 6,569,627; 6,613,560; 6,613,560 and 6,632,645; the disclosures of which are incorporated by reference in their entireties), using primer pairs that are capable of hybridizing to the proximal sequences that define or flank a polymorphic site in its double-stranded form.

10 In some embodiments of the present invention, the amplification method is itself a method for determining the identity of a polymorphic site, as for example, in allele-specific PCR. In allele-specific PCR, primer pairs are chosen such that amplification is dependent upon the input template nucleic acid containing the polymorphism of interest. In such
15 embodiments, primer pairs are chosen such that at least one primer is an allele-specific oligonucleotide primer. In some sub-embodiments of the present invention, allele-specific primers are chosen so that amplification creates a restriction site, facilitating identification of a polymorphic site. In other embodiments of the present invention, amplification of the target polynucleotide is by multiplex PCR. Through the use of multiplex PCR, a multiplicity of regions of a target polynucleotide may be amplified simultaneously. This is particularly
20 advantageous in those embodiments wherein greater than a single polymorphism is detected.

In lieu of PCR, alternative methods, such as the "Ligase Chain Reaction" ("LCR") may be used (Barany, F., Proc. Natl. Acad. Sci. (U.S.A.) 88:189-193 (1991)). The "Oligonucleotide Ligation Assay" ("OLA") (Landegren, U. *et al.*, Science 241:1077-1080 (1988)) shares certain similarities with LCR and is also a suitable method for analysis of
25 polymorphisms. Nickerson, D. A. *et al.* have described a nucleic acid detection assay that combines attributes of PCR and OLA (Nickerson, D. A. *et al.*, Proc. Natl. Acad. Sci. (U.S.A.) 87:8923-8927 (1990)). Other known nucleic acid amplification procedures, such as transcription-based amplification systems (Malek, L. T. *et al.*, U.S. Pat. No. 5,130,238; Davey, C. *et al.*, European Patent Application 329,822; Schuster *et al.*, U.S. Pat. No.
30 5,169,766; Miller, H. I. *et al.*, PCT Application WO89/06700; Kwoh, D. *et al.*, Proc. Natl. Acad. Sci. (U.S.A.) 86:1173 (1989); Gingeras, T. R. *et al.*, PCT Application WO88/10315)),

or isothermal amplification methods (Walker, G. T. *et al.*, Proc. Natl. Acad. Sci. (U.S.A.) 89:392-396 (1992)) may also be used.

5 An advantageous gene, particularly its alleles, the *ob* gene. Other genetic sequences include, but are not limited to, microsatellite loci for use in bovine parentage verification, including those designated ISAG markers, URB markers as developed by H. Lewin at the Bovine Blood Typing Lab, Saskatchewan Research Council, Saskatoon, Saskatchewan, Canada, and other species specific and genotype specific nucleotide sequences.

10 The invention also provides for the sequencing of the genetic sequences described above. Advantageously, the nucleic acid sequencing is by automated methods (reviewed by Meldrum, Genome Res. 2000 Sep;10(9):1288-303, the disclosure of which is incorporated by reference in its entirety). Methods for sequencing nucleic acids include, but are not limited to, automated fluorescent DNA sequencing (see, e.g., Watts & MacBeath, Methods Mol Biol. 2001;167:153-70 and MacBeath *et al.*, Methods Mol Biol. 2001;167:119-52), capillary electrophoresis (see, e.g., Bosserhoff *et al.*, Comb Chem High Throughput Screen. 2000 Dec;3(6):455-66), DNA sequencing chips (see, e.g., Jain, Pharmacogenomics. 2000 Aug;1(3):289-307), mass spectrometry (see, e.g., Yates, Trends Genet. 2000 Jan;16(1):5-8), pyrosequencing (see, e.g., Ronaghi, Genome Res. 2001 Jan;11(1):3-11), and ultrathin-layer gel electrophoresis (see, e.g., Guttman & Ronai, Electrophoresis. 2000 Dec;21(18):3952-64), the disclosures of which are hereby incorporated by reference in their entireties. The sequencing can also be done by any commercial company. Examples of such companies include, but are not limited to, the University of Georgia Molecular Genetics Instrumentation Facility (Athens, Georgia) or SeqWright DNA Technologies Services (Houston, Texas).

25 Advantageously, the amino acid sequencing is by automated methods. Methods for sequencing amino acids include, but are not limited to, alkylated-thiohydantoin method (see, e.g., Dupont *et al.*, EXS. 2000;88:119-31), chemical protein sequencing (see, e.g., Stoloritz, Curr Opin Biotechnol. 1993 Feb;4(1):9-13), Edman degradation (see, e.g., Prabhakaran *et al.*, J Pept Res. 2000 Jul;56(1):12-23), and mass spectrometry (see, e.g., McDonald *et al.*, Dis Markers. 2002;18(2):99-105), the disclosures of which are incorporated by reference in their entireties. Alternatively, amino acid sequences can be deduced from nucleic acid sequences. Such methods are well known in the art, e.g., EditSeq from DNASTAR, Inc.

30 The results of the analysis provide the genotype data that is associated with the individual animal from which the sample was taken. The genotype data is then kept in an

accessible database, and may or may not be associated with other data from that particular individual or from other animals.

The data obtained from genotyping individual animals is stored in a database which can be integrated or associated with and/or cross-matched to other databases. The database
5 along with the associated data allows information about the individual animal to be known through every stage of the animal's life, i.e., from conception to consumption of the animal product.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art of molecular biology.

10 Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described herein. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In addition, the materials, methods, and examples are illustrative only and are not intended to be limiting.

15 EXAMPLES

EXAMPLE 1: Improving Protein and Milk Production of Dairy Herds

A C to T transition in exon 2 of the leptin gene that results in an Arg25Cys substitution has been associated with increased fat deposition in beef cattle (see, e.g., Buchanan et al., Genet Sel Evol. 2002 Jan-Feb;34(1):105-16). The T allele is associated with
20 increased fat deposition in beef cattle and higher leptin mRNA levels in adipose tissue. As described in this Example, this same genetic variant is also present in dairy breeds.

Leptin is a hormone secreted predominantly from white adipose tissue and performs important roles in the control of body weight, feed intake, immune function and reproduction (see, e.g., Block et al., J Endocrinol. 2001 Nov;171(2):339-48; Santos-Alvarez et al., Cell
25 Immunol. 1999 May 25;194(1):6-11 and Kadokawa et al., Reprod Fertil Dev. 2000;12(7-8):405-11). The primary factors affecting plasma leptin levels include body fat mass and energy balance (see, e.g., Block et al., J Endocrinol. 2001 Nov;171(2):339-48). Leptin has been shown to regulate the immune response (see, e.g., Santos-Alvarez et al., Cell Immunol. 1999 May 25;194(1):6-11) and a delay in the recovery of leptin secretion post-partum
30 increases the delay to first ovulation in Holstein dairy cows (see, e.g., Kadokawa et al., Reprod Fertil Dev. 2000;12(7-8):405-11).

Body fat stores and energy balance change dramatically through the lactation cycle of a dairy cow. Body fat reserves play an important role in sustaining high milk production in early lactation, when energy intake is limited. In early lactation, dairy cattle are in a negative energy balance, such that energy must be drawn from the body fat of the cow to support milk production. Hence, body fat condition is increased prior to calving to provide energy stores.

To test for an association between leptin single nucleotide polymorphism (SNP) and milk productivity, 416 Holstein cows were genotyped and compared as to lactation performance data using a mixed model. Animals homozygous for the T allele produced more milk (1.5 kg/day versus CC animals) and somatic cell count linear scores, over the entire lactation. The increase in milk yield is most prominent in the first 100 days of lactation (2.44 kg/d), declining to 1.74 k/d between 101 and 200 days in lactation. Protein yield is also increased in such TT cows. The milk and protein yield advantages, observed in cows homozygous for the T allele, represent an economic advantage to dairy producers.

Using the PCR-RFLP to distinguish the alleles (see, e.g., Buchanan et al., Genet Sel Evol. 2002 Jan-Feb;34(1):105-16), individuals from six dairy breeds were genotyped. The SNP was present in all breeds examined (Table 1). Using Dairy Herd Improvement records for 416 Holstein cows and a total of 9584 observations (from 11 Saskatchewan herds; Table 2), associations between milk production, milk fat percentage, milk protein percentage, SCC linear score and leptin genotype were analyzed. Data were analyzed using a mixed model (SAS v. 8.0 for Windows, PROC MIXED); SAS Institute, Cary, North Carolina, USA) to account for the repeated observations within cow and the clustering of observations at the herd level. Model specifications included a random statement with subject equal to cow within herd and a compound symmetry covariance structure. Initial bivariate analyses were examined looking at the association between genotype and milk production outcomes. Potentially important covariates were then introduced using a manual step-wise process to produce the final model. Additional covariates included milk fat percent, milk protein percent, days in milk (DIM), lactation number, month the lactation started (for potential seasonal effects) and linear score. The main effects model was assessed for first-order interactions where genotype and one or more covariates remained in the model with $P < 0.05$. Model diagnostics included visual examination of the raw and standardized residuals (SAS User's guide: Statistics, Version 8 Edition, 2000, SAS Institute, Cary, North Carolina, USA).

The analysis demonstrated a significant impact of leptin genotype on milk yield, particularly in early lactation. Table 3 provides an estimate of the increase in milk yield of TT and TC genotypes relative to the CC genotype. Over the entire lactation, the TT genotype was associated with an increase in test-day milk yield of 1.5 kg per day versus CC. This effect was most prominent in early lactation (2.44 kg/d) declining to 1.74 kg/d between 101 and 200 DIM.

Analysis of milk composition indicated a negative impact of the T allele on milk fat percent, but an insignificant impact on milk fat yield. As a result, yield of 3.5% fat corrected milk was not significantly affected by genotype. The tests show that milk fat yield was substantially constant, with the milk fat distributed through a larger milk yield, thus reducing the milk fat percent. Table 4 illustrates the effect of a T allele at the leptin SNP on test-day milk fat percent.

Analysis of test-day milk protein indicated a significant protein yield increase in TT and TC cows. Table 5 illustrates the effect of a T allele at the leptin SNP on test-day milk protein yield. Increased milk yield in cows with a T allele is thus associated with a decline in milk fat percent without changing milk protein product percent such that protein yield, but not fat yield, is significantly increased.

The analysis also showed a significant impact of genotype at the leptin SNP on milk somatic cell count linear score. Cows homozygous for the T allele demonstrated a significant increase in somatic linear score over the entire lactation ($P = 0.002$) and within each of the early ($P = 0.018$), mid ($P = 0.04$) and late ($P = 0.033$) lactation periods (Table 6).

Liefers et al. using a different intronic SNP in the leptin gene also found an increase in milk and protein yield in Holsteins (see, e.g., Liefers et al., J Dairy Sci. 2002 Jun;85(6):1633-8). However, in their population the favored allele was very rare (1/613 homozygous) compared to the high frequency of the SNP reported herein.

The results in this Example indicate that the leptin TT genotype is associated with increased milk and protein yield, without changing the yield of milk fat. Selecting TT cows for milking herds will increase the milk and protein production of the herd while maintaining milk fat yield compared to a similar sized herd of CC cows.

Table 1 depicts the frequency of the C and T alleles at the leptin SNP.

Table 2 provides a description of 11 Saskatchewan dairy herds used in the study.

Table 3 illustrates the effect of a T allele at the leptin SNP on test-day milk yield (kg/d).

Table 4 depicts the effect of a T allele at the leptin SNP on test-day milk fat percent.

Table 5 relates to the effect of a T allele at the leptin SNP on test-day milk protein
5 yield (kg/d).

Table 6 illustrates the effect of a T allele at the leptin SNP on test-day somatic cell count linear score.

Table 1

Breed	# of Animals	T allele	C allele
Holstein	416	0.46	0.54
Ayrshire	17	0.62	0.38
Brown Swiss	21	0.45	0.55
Canadienne	9	0.11	0.89
Guernsey	16	0.06	0.94
Jersey	20	0.53	0.47

10 Table 2

Item	Mean	Minimum	Maximum
Number of milking cows	71	36	129
Herd average milk yield (kg/d)	30.5	19.0	36.8
Herd average fat percent (%)	3.68	2.94	4.51
Herd average protein percent (%)	3.22	3.01	3.44
Herd average somatic cell count (cells/mL)	300,000	81,000	518,000

Table 3

Genotype ¹	Estimate (kg/d)	Degrees of Freedom	Probability P	Lower confidence limit (kg/d)	Upper confidence limit (kg/d)
Entire lactation					
TT	1.50	9149	0.04	0.05	2.95
TC	0.91	9149	0.12	-0.24	2.07
CC	--	--	--	--	--

Continuation of Table 3

Genotype ¹	Estimate (kg/d)	Degrees of Freedom	Probability P	Lower confidence limit (kg/d)	Upper confidence limit (kg/d)
0-100 DIM ²					
TT	2.44	2499	0.004	0.78	4.11
TC	1.74	2499	0.01	0.41	3.07
CC	--	--	--	--	--
101-200 DIM					
TT	1.74	2507	0.04	0.11	3.37
TC	1.38	2507	0.04	0.08	2.68
CC	--	--	--	--	--
> 200 DIM					
TT	0.24	3299	0.729	-1.14	1.63
TC	0.22	3299	0.693	-0.88	1.32
CC	--	--	--	--	--

¹ Covariates included milk fat percent, milk protein percent, days in milk, lactation number, month that the lactation started (for potential seasonal effects), and somatic cell linear score.

² Days in milk

Table 4

Genotype ¹	Estimate (%)	Degrees of Freedom	Probability P	Lower confidence limit (%)	Upper confidence limit (%)
0-300 DIM ²					
TT	-0.10	9150	0.140	-0.24	0.03
TC	-0.07	9150	0.179	-0.18	0.03
CC	--	--	--	--	--
101-200 DIM					
TT	-0.15	2508	0.057	-0.31	0.00
TC	-0.12	2508	0.056	-0.25	0.00
CC	--	--	--	--	--

¹ Covariates included milk fat percent, milk protein percent, days in milk, lactation number, month that the lactation started (for potential seasonal effects), and somatic cell linear score.

² Days in milk

Table 5

Genotype ¹	Estimate (kg/d)	Degrees of Freedom	Probability <i>P</i>	Lower confidence limit (kg/d)	Upper confidence limit (kg/d)
0-300 DIM ²					
TT	0.043	9161	0.063	-0.002	0.090
TC	0.025	9161	0.182	-0.012	0.062
CC	--	--	--	--	--
0-100 DIM					
TT	0.072	2500	0.006	0.021	0.123
TC	0.050	2500	0.017	0.009	0.091
CC	--	--	--	--	--
101-200 DIM					
TT	0.047	2508	0.073	-0.004	0.099
TC	0.037	2508	0.074	-0.004	0.079
CC	--	--	--	--	--

¹Covariates included milk fat percent, milk protein percent, days in milk, lactation number, month that the lactation started (for potential seasonal effects), and somatic cell linear score.

²Days in milk

5

Table 6

Genotype ¹	Estimate	Degrees of Freedom	Probability <i>P</i>	Lower confidence limit	Upper confidence limit
0-300 DIM ²					
TT	0.540	9152	0.002	0.203	0.876
TC	0.230	9152	0.092	-0.037	0.498
CC	--	--	--	--	--
0-100 DIM					
TT	0.482	2513	0.018	0.083	0.881
TC	0.227	2513	0.164	-0.092	0.546
CC	--	--	--	--	--
101-200 DIM					
TT	0.517	2507	0.040	0.120	0.913

Continuation of Table 6

Genotype ¹	Estimate	Degrees of Freedom	Probability <i>P</i>	Lower confidence limit	Upper confidence limit
TC	0.099	2507	0.040	-0.218	0.415
CC	--	--	--	--	--
Plus 200 DIM					
TT	0.386	3313	0.033	0.032	0.740
TC	0.140	3113	0.330	-0.142	0.422
CC	--	--	--	--	--

¹Covariates included milk fat percent, milk protein percent, days in milk, lactation number, month that the lactation started (for potential seasonal effects), and somatic cell linear score.

²Days in milk

5 EXAMPLE 2: Distribution of Leptin Genotype in Guelph Cows

The objective of this Example is to examine the relationship between leptin genotype and milk production in approximately 1000 mixed age dairy cows from farms in Canada.

A hair sample is collected from each animal and the specific leptin genotype is ascertained. The result of each cow will be matched against its individual performance data
 10 for milk yield, somatic cell count, milk fat and milk protein. Any associations between animal leptin genotype and milk production or composition will be evaluated and reported.

All genotype results have been reported back to the investigators and descriptive statistics of the frequency distribution of genotypes were prepared (see Table 7).

Table 7 depicts the percent distribution of leptin genotypes in Guelph cows.

15 Table 7

Genotype	Number	Percent (%)
CC	171	33.7
CT	262	51.7
TT	74	14.6
Total	507	100.0

EXAMPLE 3: Relationship Between Leptin Genotype, Milk Production And Energy Balance

The objective of this Example is to examine the relationship between leptin genotype, milk production and energy balance during the peri-parturient period.

Milk yield, dry matter intake and various metabolic hormones were measured to
 20 assess the effect of leptin genotype on energy balance in dairy cows. There is evidence in

some cows for increased milk yield in TT cows as well as non-esterified fatty acid (NEFA) and beta-hydroxy butyrate (BHBA) levels indicating differences between cows in energy balance level associated with genotype (see, e.g., FIGS. 1 and 2). Cows that produce more milk should eat more, in which case they will not have effects on NEFA and BHBA. Conversely, if those cows milk more but do not eat any more, they will have elevated NEFA and BHBA levels.

The energy balance in the dairy cow is related to dry matter intake (DMI). The DMI drops 32% in the last three weeks prepartum and 90% of that drop is in the last week. Heifers eat less than cows in an absolute sense (lbs/day) and in a relative sense (% body weight). Diet composition, BCS (Body Condition Score or the amount of fat on the animal), parity and time from calving together explain only 18% of cow-to-cow variation in prepartum DMI.

A dairy cow faces metabolic challenges during the transition from prepartum to postpartum. Decreased DMI and increased output of energy, vitamins and minerals lead to negative energy balance (NEB) and hypocalcemia which results in immunosuppression. Burning NEFA in liver may inhibit DMI and probable roles of insulin (especially postpartum) and leptin decreases DMI. Decreased DMI is part of the fatty liver syndrome, in which decreased DMI leads to increased NEB, which results in increased fat mobilization, leading to increased NEFA, which results in increased ketosis and fat accumulation in liver, which leads to decreased DMI. If DMI is maintained throughout transition (see, e.g., Bertics et al., J Dairy Sci. 1992 Jul;75(7):1914-22) or if energy intake is supplemented (see, e.g., Studer et al., J Dairy Sci. 1993 Oct;76(10):2931-9), then there is a decrease in liver fat accumulation.

High-producing dairy cows inevitably experience a period of negative-energy balance during early lactation. The extent and duration of postpartum negative energy balance has been related to an increased incidence of metabolic disorders, and decreased reproductive efficiency. Cows that undergo severe and/or prolonged energy deficits are at increased risk of subclinical ketosis and several other health problems (see, e.g., Duffield, Vet Clin North Am Food Anim Pract. 2000 Jul;16(2):231-53). In addition, the role of negative energy balance in the etiology of displaced abomasums in postparturient dairy cows has recently been reviewed (see, e.g., Geishauser et al., Vet Clin North Am Food Anim Pract. 2000 Jul;16(2):255-65).

A great deal of research and extension has been expended to develop and implement strategies for improvement of negative energy balance in periparturient dairy cows. Most dairy producers have gone to considerable effort to organize transition management and nutritional programs for their cows immediately before and after calving. In addition, supplementation programs are widely used to improve energy sources or utilization during this period. A monensin controlled release capsule is available for prevention of subclinical ketosis and displaced abomasums (see, e.g., Duffield et al., J Dairy Sci. 1998 Sep;81(9):2354-61 and Duffield et al., J Dairy Sci. 1998 Nov;81(11):2866-73). Rumen-protected choline is commonly used in transition cow diets to improve liver metabolism and energy utilization (see, e.g., Erdman & Sharma, J Dairy Sci. 1991 May;74(5):1641-7). Propylene glycol is used for both therapy and prevention of subclinical ketosis (see, e.g., Studer et al., J Dairy Sci. 1993 Oct;76(10):2931-9). All of these strategies are quite successful in assisting dairy producers to manage their cows through this stressful period of the lactation cycle. It is interesting to note that there is wide variability between cows in the success of these strategies (see, e.g., Duffield, Vet Clin North Am Food Anim Pract. 2000 Jul;16(2):231-53). The reasons for this variability are far from fully elucidated.

Leptin is a naturally occurring hormone secreted by adipocytes and is involved in the control of energy balance (see, e.g., Liefers et al., Mamm Genome. 2003 Sep;14(9):657-63). The production of leptin is controlled by a single gene location. Leptin concentrations in the blood affect the regulation of food consumption and energy expenditure. As leptin concentrations rise, the body responds by reducing appetite and increasing metabolism (see, e.g., Liefers et al., Mamm Genome. 2003 Sep;14(9):657-63). However, there is a lack of information on the control of these mechanisms. In a recent report, it was concluded that plasma leptin was regulated by nutrition in early postnatal life, but that a sudden increase in plasma leptin is not required for the onset of puberty in dairy cattle (see, e.g., Block et al., J Dairy Sci. 2003 Oct;86(10):3206-14).

Body fat reserves play an important role in sustaining milk production in early lactation, when energy intake is limiting. Allelic variation (C to T transition) in the leptin gene has been associated with increased fat deposition in beef cattle (see, e.g., Buchanan et al., Genet Sel Evol. 2002 Jan-Feb;34(1):105-16). The T allele was associated with increased fat deposition and higher leptin mRNA levels in adipose tissue. In a recent study, animals that were homozygous for the T allele produced more milk, had higher somatic cell count

linear scores, without significantly affecting milk fat or protein percent over the entire lactation (see, e.g., Buchanan et al., J Dairy Sci. 2003 Oct;86(10):3164-6). Thus, leptin genotype testing could provide considerable insight into the metabolic status and milk production potential of dairy cattle, as well as to guide dairy producers with selection, breeding and herd management decisions (see, e.g., Buchanan et al., J Dairy Sci. 2003 Oct;86(10):3164-6).

Recently, the Igenity-L genotype test has become commercially available. This test determines the status of an individual animal for the C and T leptin alleles. The test is performed on DNA extracted from hair follicles.

The association between the leptin genotype and various aspects of periparturient metabolism and performance in dairy cattle are to be determined. It is hypothesized that leptin allele type will be associated with dry matter intake, metabolic indicators of energy balance in blood, liver metabolism, general health and production in periparturient dairy cattle.

The objectives are to (1) determine the associations between leptin genotype, dry matter intake, health and production; (2) to study the relationships of leptin genotype with metabolic function and liver concentrations of glycogen and triglyceride in the periparturient period and (3) to evaluate interactions between the effect of supplementation with monensin, choline or propylene glycol and leptin genotype.

The experimental animals will be from two distinct populations of dairy cows that have been intensively studied through their involvement in separate transition cow research efforts. Both populations were used to study the effect of specific intervention protocols on the metabolism and productivity of the cows involved. For each of the populations, very similar (but not exactly the same) outcome variables, have been collected. For example, one of the populations had serum profiles taken only in the postpartum period. Also, a subset of animals in one of the populations had liver biopsies taken at calving and a Day 28 postpartum. The details of the outcome variables will be described later. The two distinct populations were obtained following their participation in the specific experiments as follows.

In Experimental Group A, approximately 180 primiparous and multiparous Holstein animals at the University of Guelph Elora and Posenby dairy research centers were used to conduct this study. Cows were housed in a tie-stall barn from three weeks prior to expected

calving date. The animals were moved to maternity pens for calving, and then transferred back to the tie-stall barn for the duration of the lactation. Cows were fed a total mixed ration (TMR) twice daily and were milked twice daily. Disease treatment protocols and complete diet information were provided. Cows were in good general health at the time of enrollment.

5 At enrollment, between day 24 and day 21 prior to expected calving date, each cow or heifer was randomly assigned to one of four treatment groups: rumen-protected choline top-dress or nothing, and/or a monensin controlled release capsule. For the choline-supplemented animals, feed was top-dressed every day thereafter until 28 days postpartum. Staff at Elora Daily Research Station were responsible for administration of the monensin controlled-release capsule and delivery of the rumen protected choline top-dress; however, were not
10 involved in any of the assessment or data collection.

In Experimental Group B, approximately 45 Holstein cows at the Pennsylvania State University dairy herd were used to conduct this study. After calving, cows went into the tiestall barn and were fed the herd TMR for fresh cows. Each cow was randomly assigned to
15 one of three treatment groups. One group received the dry propylene glycol product (Propylene 65 at a rate of 250 g/day) in the TMR. The second group received the same rate of dried propylene glycol supplementation, but as a top-dress on their TMR. Cows in the third group were assigned to be non-supplemented control cows. Milk production was measured for three weeks during which time weekly milk composition (AM and PM samples) were
20 measured (to start no earlier than three days after calving. Milk Ketone analyses were conducted at the same time milk was sampled. Cows were allocated to treatment such that previous 305 day ME (Mature Equivalent lactation record), calving date, lactation number, days dry and body condition score is balanced across treatments. Body condition was evaluated at dry off, and weekly after calving. Blood was collected for serum metabolic
25 profile analysis at 4 days after calving, and weekly until three weeks after calving. All health records of cows were recorded. Milk production was measured daily up to 56 days in milk.

The samples and measurements collected for each of the major outcome variables is as follows.

For serum metabolic profiles, blood samples were taken from the coccygeal vein and
30 tested for a serum metabolic profile, which includes beta-hydroxybutyrate (BHB), glucose, non-esterified fatty acids (NEFA), urea, aspartate transaminase (AST) and cholesterol. Blood samples were taken at enrollment, one week prior to expected calving, and at one and two

weeks post calving. Blood serum were stored by freezing at -20 C. Batches of serum were submitted to the University of Guelph Animal Health Laboratory for analysis on a periodic basis.

5 For milk ketones, milk samples were collected during week one and week two following calving. Ketone levels will be determined immediately following sample collection using the KetoTest cowside ketone test.

Body condition scores (BCS) were determined at the time of enrollment and two weeks after calving (day +8 to +14) for each animal using a five-point scale with quarter point intervals.

10 Weekly milk production was measured using automated milk recording equipment in the milking parlour. Milk weights will be collected on a daily basis from calving until Day 60 postpartum. A weekly average was taken.

15 For milk components and somatic cell counts, both yield and percentage composition of milk butterfat and protein were determined from samples collected for Ontario DHI records. This is done on an approximately 30 days test date interval. In addition, milk somatic cell counts (SCC) were determined on the source samples. These data will be collected for the first two test dates postpartum.

20 Liver biopsies were performed on approximately 60 cows in Experiment A (15 randomly selected animals from each treatment group) to determine glycogen and triglyceride content. These biopsies were taken by a trained technical associate during the first and fourth weeks after calving.

Disease occurrences and treatments were recorded in the animal's lifetime record. Standard disease protocols were followed.

25 Statistical analyses will be conducted using the Statistical Analysis Software (SAS). Descriptive statistics will be determined by comparing the differences between the three leptin allele groups. Multivariable regression analysis in SAS will be used to determine the associations between individual cow leptin allele status and the biological performance and metabolic outcome variables, while controlling for the effects of the energy status enhancement strategies that were evaluated in Experiments A and B, respectively.

30 **EXAMPLE 4: Relationship Between Leptin Genotype, Milk Production, Milk Components And Dry Matter Intake**

The objective of this Example is to examine the relationship between leptin genotype, milk production, milk components and dry matter intake in cows during the peri-parturient period.

Production data from 80 cows was retrospectively assessed for any associations with leptin genotype.

Preliminary data suggests an effect of leptin genotype on dry matter intake up to 4 weeks post-calving, and an effect on milk production, value per lactation, milk fat content and milk protein content. This data will be evaluated for its effect on energy balance and changes recommended to ration formulation in order that diet can most appropriately be linked to individual production.

Table 8 provides dry matter intake (DMI) in pounds (weeks before freshening and after freshening).

Table 9 depicts ME (305-day Mature Equivalent) lactation production by genotype and lactation number.

Table 10 relates to average all lactation ETA (Estimated Transmitting Ability).

Table 11 illustrates first lactation ETA (Estimated Transmitting Ability).

Table 12 provides for less than 200 days in milk (test day model (TDM) = residuals from the test day model, milk is just the raw average).

Table 13 relates to less than 100 days in milk (TDM = residuals from the test day model, milk is just the raw average).

Table 8

Week	CC-DMI	CC-n	CT-DMI	CT-n	TT-DMI	TT-n
-4	30.98	22	29.71	22	30.57	13
-3	31.89	30	31.03	30	31.51	20
-2	31.05	36	29.88	36	31.42	26
-1	26.04	36	25.27	36	27.87	27
1	29.32	30	31.86	30	35.04	20
2	36.92	30	37.35	30	38.79	20
3	40.73	30	41.43	30	42.60	20
4	43.07	30	43.55	30	44.84	20
5	44.71	23	42.75	23	42.15	13
6	46.31	23	43.52	23	45.55	13

Continuation of Table 8

Week	CC-DMI	CC-n	CT-DMI	CT-n	TT-DMI	TT-n
7	48.47	23	45.35	23	43.47	13
8	48.76	23	46.19	23	45.36	13
9	48.74	23	48.58	23	45.74	13

Table 9

Test	Lact	MEM	MEF	MEP	Count
CC	1	34665	1176	960	22
CC	2	30045	915	783	19
CC	3	29620	950	785	12
CC	4	29722	947	758	7
CT	1	33726	1222	965	32
CT	2	30308	1093	851	29
CT	3	29592	1053	804	21
CT	4	27544	954	692	15
TT	1	33569	1234	963	13
TT	2	31820	1052	801	12
TT	3	29918	1061	825	9
TT	4	25819	974	740	4

MEM: Mature Equivalent Milk production

MEF: Mature Equivalent Fat production in kilograms

MEP: Mature Equivalent Protein production in kilograms

Count: Somatic Cell Count (1000 cells/mL of milk)

It should be noted that MEM, MEF, MEP and Count are all measures of the production of milk or milk component for a hypothetical 305-day lactation period, or the average cell count on a test day for somatic cell count.

Table 10*

Test	\$\$\$ ¹	Milk ²	Fat ³	Protein ⁴	Count ⁵
CC	182	1522	25	39	22
CT	196	1454	32	40	33
TT	218	1634	36	45	13

¹estimated value of the production of the animal in comparison to breed averages

²estimated additional milk production per lactation for test cows in comparison to breed average

³estimated additional fat production for test cows in comparison to breed average

⁴estimated additional protein production for test cows in comparison to breed average

⁵average somatic cell count for test cows (1000 cells/mL)

*the figures shown in this table are quoted for all lactations

Table 11*

Test	\$\$\$ ¹	Milk ²	Fat ³	Protein ⁴	Count ⁵
CC	195	1635	29	41	21
CT	188	1393	32	38	30
TT	222	1608	35	46	12

¹estimated value of the production of the animal in comparison to breed averages

²estimated additional milk production per lactation for test cows in comparison to breed average

³estimated additional fat production for test cows in comparison to breed average

⁴estimated additional protein production for test cows in comparison to breed average

⁵average somatic cell count for test cows (1000 cells/mL)

*the figures shown in this table are quoted for 1st lactation cows.

Table 12

Test	Lact	TDM ¹ Milk	TDM Fat	TDM Pro	TDM RSCC ²	Milk	Fat %	Pro %	SCC ³	Count
CC	1	0.59	-0.194	-0.48	-0.219	82	3.33	2.41	1.63	115
CC	2	4.76	-0.026	0.052	-0.072	99	3.20	2.43	2.21	78
CC	3	11.25	0.377	0.101	0.262	112	3.26	2.58	3.12	44
CC	4	12.16	0.004	0.162	0.948	109	3.41	2.68	4.64	23
CT	1	-1.11	-0.153	-0.0028	-0.051	81	3.56	2.44	1.91	170
CT	2	-0.72	0.061	0.022	-0.025	91	3.62	2.25	2.35	157
CT	3	1.69	-0.581	-0.043	-0.373	102	3.61	2.64	2.06	98
CT	4	2.78	0.152	0.045	-0.496	104	3.83	2.60	2.35	46
TT	1	-0.51	-0.017	0.053	-0.084	80	3.62	2.48	1.86	74
TT	2	3.62	-0.062	0.019	-0.322	98	3.60	2.60	1.80	53
TT	3	-2.21	0.544	-0.038	-0.738	96	3.82	2.75	1.67	38
TT	4	-14.21	0.081	-0.237	0.400	87	3.92	2.11	3.50	14

¹test day model

²residual of somatic cell count

³somatic cell counts

Table 13

Test	Lact	TDM ¹ Milk	TDM Fat	TDM Pro	TDM SCC	Milk	Fat %	Pro %	SCC ²	Count
CC	1	-1.50	-0.079	-0.098	-0.113	79	3.38	2.10	1.81	54
CC	2	-0.46	-0.244	-0.098	-0.209	97	3.31	2.19	1.83	41
CC	3	9.54	0.210	-0.044	0.212	113	3.45	2.49	2.70	23
CC	4	15.46	-0.275	0.210	0.974	115	3.49	2.66	4.62	15
CT	1	-3.32	-0.028	-0.090	-0.149	773	3.65	2.03	1.81	81
CT	2	-0.89	-0.125	0.006	0.038	943	3.70	1.78	2.23	81
CT	3	-1.32	0.391	-0.208	-0.411	102	3.76	2.53	1.92	49
CT	4	0.82	-0.047	-0.136	-0.450	103	4.08	2.41	2.59	27
TT	1	-2.16	0.027	0.046	0.102	76	3.81	2.14	2.14	35
TT	2	3.33	-0.169	-0.050	-0.082	100	3.96	2.47	2.03	27
TT	3	-1.90	0.321	-0.106	-0.858	99	3.92	2.59	1.32	21
TT	4	-1.69	-0.125	-0.087	-0.217	94	3.70	1.38	2.40	8

¹TDM = test day model²SCC = somatic cell counts

* * *

5 The invention is further described by the following numbered paragraphs:

1. A composition for the detection of *ob* gene polymorphisms, comprising at least one oligonucleotide consisting essentially of a nucleic acid sequence which complements and specifically hybridizes to an *ob* gene nucleic acid molecule, wherein the sequence is at least 80% homologous to a sequence selected from the group consisting of
10 SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:7.

2. A composition for the detection of *ob* gene polymorphisms, comprising at least one oligonucleotide consisting essentially of a nucleic acid sequence which complements and specifically hybridizes to an *ob* gene nucleic acid molecule, wherein the sequence is selected from the group consisting of SEQ ID NO:4, SEQ ID NO:5, SEQ ID
15 NO:6, and SEQ ID NO:7, and a nucleotide sequence which differs from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:7 by a one base change or substitution therein.

3. An isolated and purified oligonucleotide primer pair for enzymatic amplification of *ob* gene DNA, comprising a pair of nucleic acid sequences which complement and specifically hybridize to an *ob* gene nucleic acid molecule, wherein the pair

of nucleic acid sequences is at least 95% homologous to sequences selected from the group consisting of (a) the oligonucleotide pair of SEQ ID NO:4 and SEQ ID NO:5 and (b) the oligonucleotide pair of SEQ ID NO:6 and SEQ ID NO:7.

4. An isolated and purified oligonucleotide primer pair for enzymatic
5 amplification of *ob* gene DNA, comprising a pair of nucleic acid sequences which complement and specifically hybridize to an *ob* gene nucleic acid molecule, wherein the pair of nucleic acid sequences is selected from the group consisting of (a) the oligonucleotide pair of SEQ ID NO:4 and SEQ ID NO:5, and (b) a nucleotide pair which differs from SEQ ID NO:4 and SEQ ID NO:5 by a one base change or substitution therein.

10 5. An oligonucleotide primer for identifying bovine having an *ob* gene polymorphism, the primer comprising at least 10 nucleotides in length and which includes at least nine contiguous nucleotides of a sequence selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:5.

15 6. An oligonucleotide probe for identifying bovine having an *ob* gene polymorphism, the probe comprising at least 10 nucleotides in length and which includes at least nine contiguous nucleotides of a sequence selected from the group consisting of SEQ ID NO:6 and SEQ ID NO:7.

7. The composition of paragraph 1, 2, 3, 4, 5 or 6 wherein the oligonucleotide is labeled with a detectable moiety.

20 8. The composition of paragraph 7 wherein the detectable moiety is selected from the group consisting of a digoxigenin-dUTP, biotin, calorimetric, fluorescent, chemiluminescent, electrochemiluminescent signal and a radioactive component.

9. The composition of paragraph 7, wherein the detectable moiety is a
fluorescent component generating a fluorescent signal.

25 10. The composition of paragraph 5 wherein the primer is from about 10 to about 24 bases in length.

11. The composition of paragraph 6 wherein the primer is from about 14 to about 30 bases in length.

30 12. The composition of paragraph 5 wherein the primer is immobilized on a solid support.

13. An oligonucleotide microarray having immobilized thereon a plurality of probes, wherein at least one of said probes is specific for the variant form of the single nucleotide polymorphism at position 189 of SEQ ID NO:1.

14. An oligonucleotide microarray having immobilized thereon a plurality of probes, wherein at least one of said probes is specific for the reference form of the single nucleotide polymorphism at position 189 of SEQ ID NO: 1.

15. The microarray of paragraph 13 wherein the probe is a nucleic acid sequence which complements and specifically hybridizes to an *ob* gene nucleic acid molecule, wherein the sequence is selected from the group consisting of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:7, and a nucleotide sequence which differs from SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:7 by a one base change or substitution therein.

16. A method for analyzing or determining polymorphism or mutation of a target nucleic acid or gene, which comprises hybridizing a nucleic acid probe according to paragraph 7 to the target nucleic acid or gene, and measuring a change in detectable moiety.

17. A method for analyzing or determining polymorphism or mutation of a target nucleic acid or gene, which comprises hybridizing a nucleic acid probe according to paragraph 9 to the target nucleic acid or gene, and measuring a change in fluorescence.

18. A method of detecting the presence of *ob* gene polymorphisms in a nucleic acid sample comprising: comprising (a) contacting the target nucleic acid of interest with at least one sensor oligonucleotide, wherein the sensor oligonucleotide comprises a sequence complementary to at least a portion of the target nucleic acid of interest, wherein the sensor oligonucleotide hybridizes to the target nucleic acid at a position suspected of containing the *ob* gene polymorphism and (b) subjecting the captured target nucleic acid and hybridized sensor probe oligonucleotide to destabilizing conditions, wherein the destabilizing conditions are sufficient to cause the sensor oligonucleotide to dissociate under differing conditions depending upon the presence of the cc, ct or tt polymorphisms in the *ob* gene.

19. The method of paragraph 18 wherein the method further comprises (c) detecting the hybridization of the sensor oligonucleotide to the target nucleic acid under the varying destabilizing conditions, whereby the presence of the specific sequence in the target nucleic acid is determined.

20. The method of paragraph 18 wherein the method further comprises a preparatory step of amplifying one or more target nucleic acid sequences from the nucleic acids of a sample, wherein the amplicons become the target nucleic acids.

21. The method of paragraph 20 wherein the amplification step produces single
5 stranded amplicons, which are then utilized as the single stranded target nucleic acids.

22. The method of paragraph 20 wherein the amplification step produces double stranded amplicons, further comprising a step of subjecting the amplicons to denaturing conditions to form single stranded target nucleic acids.

23. The method of paragraph 20 wherein the amplification step is by an
10 amplification method selected from the group consisting of polymerase chain reaction (PCR), strand displacement amplification (SDA), nucleic acid sequence-based amplification (NASBA), rolling circle amplification, T7 mediated amplification, T3 mediated amplification, and SP6 mediated amplification.

24. The method of paragraph 18 wherein the detection of the hybridization of the
15 sensor oligonucleotide is by the detection of a labeling moiety on the sensor oligonucleotide selected from the group consisting of fluorescent moieties, bioluminescent moieties, chemiluminescent moieties, and colorigenic moieties. Advantageously, the labeling moiety is a fluorescent moiety selected from the group consisting of fluorescein derivatives, BODIPYL dyes, rhodamine derivatives, Lucifer Yellow derivatives, and cyanine (Cy) dyes.

20 25. The method of paragraph 18 wherein the destabilizing conditions are created by methods selected from the group consisting of making temperature adjustments, making ionic strength adjustments, making adjustments in pH, and combinations thereof.

26. A method of detecting the presence of *ob* gene polymorphisms in a nucleic acid sample comprising: (a) contacting a single stranded target nucleic acid of interest with
25 (i) a first sensor oligonucleotide, wherein the first sensor oligonucleotide comprises a sequence complementary to at least a portion of the target nucleic acid of interest; (ii) further contacting the target nucleic acid with at least a second sensor oligonucleotide, wherein the second sensor oligonucleotide comprises a sequence complementary to at least a portion of the target nucleic acid of interest; (b) subjecting the target nucleic acid and hybridized sensor
30 oligonucleotides to destabilizing conditions, wherein the destabilizing conditions are sufficient to cause the first and/or second sensor oligonucleotide to dissociate under different destabilizing conditions; and (c) detecting the hybridization of the first and second sensor

oligonucleotide to the target nucleic acid, whereby the presence of the polymorphism in the target nucleic acid is determined.

27. The method of paragraph 1 wherein the first and second sensor oligonucleotides are differently labeled with first and second labeling moieties.

5 28. A method of detecting the presence of *ob* gene polymorphisms in a nucleic acid sample comprising: a) contacting the sample with a hybridization probe comprising one or more oligonucleotides of at least 10 nucleotides in length comprising at least nine contiguous bases of the sequences selected from the group consisting of SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:7, labeled with a detectable moiety, under suitable
10 conditions permitting hybridization of the labeled oligonucleotide probe to the *ob* gene nucleic acid to form a hybridization complex, and b) detecting the presence of the probe bound to the nucleic acid sequences by detecting the detectable moiety of the labeled oligonucleotide probe hybridized to the *ob* gene polymorphism sequences.

29. A method of detecting the presence of *ob* gene polymorphism in a nucleic acid
15 sample comprising: a) obtaining a nucleic acid molecule sample containing an *ob* gene polymorphism from a subject; b) amplifying a region of the *ob* gene polymorphism using the oligonucleotide pair of SEQ ID NO:4 and SEQ ID NO:5 to form nucleic acid amplification products; c) contacting the amplified *ob* gene polymorphism sequences from step (b), if present, with hybridization probes comprising the oligonucleotide pair of SEQ ID NO:6 and
20 SEQ ID NO:7, labeled with a detectable moiety under suitable conditions permitting hybridization of the labeled oligonucleotide probe to amplified *ob* gene polymorphism sequences to form a hybridization complex, and d) detecting the presence of amplified *ob* gene polymorphism sequences by detecting the detectable moiety of the labeled oligonucleotide probe hybridized to the amplified *ob* gene polymorphism sequences.

25 30. The methods of paragraphs 29, wherein the method further comprises, after contacting the *ob* gene polymorphism sequences with hybridization probes, subjecting the hybridized complex structures to destabilizing conditions sufficient to cause the probes to dissociate from the complex structures if there is at least one base-pair mismatch between the probes and the target nucleic acids or amplification products, and detecting a loss or a
30 retention of the probes from the hybridization complex.

31. The method of paragraph 29 wherein the amplification step is by an amplification method selected from the group consisting of polymerase chain reaction (PCR),

strand displacement amplification (SDA), nucleic acid sequence-based amplification (NASBA), rolling circle amplification, T7 mediated amplification, T3 mediated amplification, and SP6 mediated amplification.

32. The method of paragraph 29 wherein the method comprises a step of
5 subjecting the target nucleic acids of the sample to denaturing conditions to form single stranded target nucleic acids.

33. The method of paragraph 29 wherein the detection of the hybridization of the sensor oligonucleotide is by the detection of a labeling moiety on the sensor oligonucleotide selected from the group consisting of fluorescent moieties, bioluminescent moieties,
10 chemiluminescent moieties, and colorigenic moieties. Advantageous, the labeling moiety is a fluorescent moiety selected from the group consisting of fluorescein derivatives, BODIPYL dyes, rhodamine derivatives, Lucifer Yellow derivatives, and cyanine (Cy) dyes.

34. The method of paragraph 29 wherein the destabilizing conditions are created by methods selected from the group consisting of making temperature adjustments, making
15 ionic strength adjustments, making adjustments in pH, and combinations thereof.

35. The method of paragraph 29, wherein the presence of the amplified *ob* gene polymorphism sequences hybridized to labeled oligonucleotide probe correlates to the subject's propensity to deposit fat.

36. The method of paragraph 29, wherein the amplified DNA sequences are from
20 the *ob* region of the *Bos taurus* genome.

37. The method of paragraph 29, additionally comprising adding an internal standard for accessing relative amounts of DNA after amplification.

38. The method of paragraph 29, wherein presence of the amplified *ob* gene polymorphism sequences hybridized to labeled oligonucleotide probe is correlated to the
25 presence of an *ob* gene polymorphism in the sample by comparing the amount of amplification product to the quantity of amplification products formed from known internal standards.

39. The method of paragraph 29, wherein the primers comprise the oligonucleotide pair of SEQ ID NO:4 and SEQ ID NO:5.

30 40. The method of paragraph 29, wherein the detectable moiety is selected from the group consisting of a digoxigenin-dUTP, biotin, calorimetric, fluorescent, chemiluminescent, electrochemiluminescent signal and a radioactive component.

41. The method of paragraph 29, wherein the detectable moiety is a fluorescent component generating a fluorescent signal.

42. A method of selecting livestock comprising the steps of: a) obtaining a nucleic acid molecule sample containing an *ob* gene polymorphism from livestock; b) 5 amplifying a region of the *ob* gene polymorphism using the oligonucleotide pair of SEQ ID NO:4 and SEQ ID NO:5 to form nucleic acid amplification products; c) contacting the amplified *ob* gene polymorphism sequences from step (b), if present, with hybridization probes comprising the oligonucleotide pair of SEQ ID NO:6 and SEQ ID NO:7, labeled with a detectable moiety under suitable conditions permitting hybridization of the labeled 10 oligonucleotide probe to amplified *ob* gene polymorphism sequences to form duplex structures, d) detecting the presence of amplified *ob* gene polymorphism sequences by detecting the detectable moiety of the labeled oligonucleotide probe hybridized to the amplified *ob* gene polymorphism sequences; and e) identifying those livestock animals having a greater or lesser milk productivity based on the detection.

15 43. A diagnostic test kit for detection of an *ob* gene polymorphism comprising: (a) at least one oligonucleotide primer pair selected from the group consisting of the oligonucleotide pair of SEQ ID NO:4 and SEQ ID NO:5, and (b) at least one oligonucleotide probe labeled with a detectable moiety selected from the group consisting SEQ ID NO:6 and SEQ ID NO:7.

20 44. The diagnostic test kit of paragraph 43, further comprising at least one additional reagent selected from the group consisting of a lysing buffer for lysing cells contained in the specimen; enzyme amplification reaction components dNTPs, reaction buffer, and amplifying enzyme; and a combination thereof.

25 45. The diagnostic kit of paragraph 43, wherein the primers comprise the oligonucleotide pair of SEQ ID NO:4 and SEQ ID NO:5.

46. The diagnostic kit of paragraph 43, wherein the hybridization probes comprise SEQ ID NO:6 and SEQ ID NO:7.

30 47. The diagnostic kit of paragraph 43, wherein the hybridization probe further comprises a detectable moiety selected from the group consisting of a chemiluminescent component, a fluorescent component, and a radioactive component.

48. A method of increasing milk production in a selected group of livestock animals of the same species comprising:

(i) determining a genetic predisposition of each animal to produce milk by determining their *ob* genotype; and

(ii) selecting animals that possess the T-containing allele of the *ob* gene for inclusion in the group.

5 49. The method of paragraph 48 wherein increasing milk production in a selected group of livestock animals of the same species occurs during the first one hundred days of lactation.

 50. The method of paragraph 49 wherein determining comprises determining whether the animal is a TT animal homozygous with respect to the T-allele of the *ob* gene, a
10 CC animal homozygous with respect to the C-allele of the *ob* gene, or a CT animal heterozygous with respect to the T-allele and the C-allele of the *ob* gene.

 51. A method of paragraph 50 wherein selecting is selecting from the group consisting of TT animals homozygous with respect to the T-allele of the *ob* gene and CT animals heterozygous with respect to the T-allele and the C-allele of the *ob* gene.

15 52. A method of identifying those animals having increased feed conversion efficiency compared to general population of animals of the same species by determining their *ob* genotype wherein animals that possess the T-containing allele of the *ob* gene have an increased feed conversion efficiency compared to animals that possess only the C-containing allele of the *ob* gene.

20 53. A method of paragraph 52 wherein TT animals homozygous with respect to the T-allele of the *ob* gene have a greater milk productivity than CT animals heterozygous with respect to the T-allele.

 54. A method of breeding livestock animals to increase milk production in the offspring comprising selecting breeding pairs of livestock animals of the same species to
25 increase occurrence of the *ob* T-allele in the offspring.

 55. The method of paragraph 54 wherein the milk production is increased in the first one hundred days of lactation in the offspring.

 56. A method of increasing milk production in a selected group of livestock animals of the same species comprising:

30 (a) determining a genetic predisposition of each animal to produce milk by determining their *ob* genotype;

- (a) selecting animals that possess the T-containing allele of the *ob* gene for inclusion in the group; and
- (b) increasing the amount of feed for in the selected group.

57. The method of paragraph 56 wherein increasing milk production in a selected
5 group of livestock animals of the same species occurs during the first one hundred days of lactation.

58. The method of paragraph 57 wherein determining comprises determining whether the animal is a TT animal homozygous with respect to the T-allele of the *ob* gene, a CC animal homozygous with respect to the C-allele of the *ob* gene, or a CT animal
10 heterozygous with respect to the T-allele and the C-allele of the *ob* gene.

59. A method of paragraph 58 wherein selecting is selecting from the group consisting of TT animals homozygous with respect to the T-allele of the *ob* gene and CT animals heterozygous with respect to the T-allele and the C-allele of the *ob* gene.

60. The method of any one of the paragraphs 48 to 59 wherein the livestock
15 animal is a bovine, an ovine, an avian or a swine.

61. The method of paragraph 60 wherein the livestock animal is a bovine.

62. The method of paragraph 61 wherein the bovine is a dairy cattle.

* * *

Having thus described in detail advantageous embodiments of the present invention, it
20 is to be understood that the invention defined by the above paragraphs is not to be limited to particular details set forth in the above description as many apparent variations thereof are possible without departing from the spirit or scope of the present invention.

WHAT IS CLAIMED IS:

1. A method of identifying those animals having greater milk productivity from a group of livestock animals of the same species comprising:

(c) selecting the livestock, wherein the selecting comprises:

- 5 (i) obtaining a nucleic acid molecule sample containing an *ob* gene polymorphism from livestock,
- (ii) amplifying a region of the *ob* gene polymorphism with the oligonucleotide pair of SEQ ID NO:4 and SEQ ID NO:5 to form nucleic acid amplification products,
- 10 (iii) contacting the amplified *ob* gene polymorphism sequences from step (ii), with hybridization probes consisting essentially of the oligonucleotide pair of SEQ ID NO:6 and SEQ ID NO:7, labeled with a detectable moiety under suitable conditions permitting hybridization of the labeled oligonucleotide probe to
- 15 amplified *ob* gene polymorphism sequences to form duplex structures,
- (iv) detecting the presence of amplified *ob* gene polymorphism sequences by detecting the detectable moiety of the labeled oligonucleotide probe hybridized to the amplified *ob* gene
- 20 polymorphism sequences, and
- (v) selecting the type of the livestock animal based on the detection of the *ob* gene polymorphism; and
- (b) identifying those animals having a greater milk productivity based on the presence of a particular *ob* gene polymorphism.

25 2. The method of claim 1 wherein the selecting comprises determining whether the livestock animal is a TT animal homozygous with respect to the T-allele of the *ob* gene, a CC animal homozygous with respect to the C-allele of the *ob* gene, or a CT animal heterozygous with respect to the T-allele and the C-allele of the *ob* gene.

30 3. A method of claim 1 wherein the selecting is selecting from the group consisting of TT animals homozygous with respect to the T-allele of the *ob* gene and CT animals heterozygous with respect to the T-allele and the C-allele of the *ob* gene to select those animals having a greater feed conversion efficiency.

4. The method of claim 1 wherein the *ob* gene polymorphism is a C to T transition that results in Arg25Cys.
5. The method of claim 1 wherein the livestock animal is a bovine, an ovine, an avian or a swine.
- 5 6. The method of claim 5 wherein the livestock animal is a bovine.
7. The method of claim 6 wherein the bovine is dairy cattle.
8. A method of increasing milk production in a selected group of livestock animals of the same species comprising:
- 10 (a) determining a genetic predisposition of each animal to produce milk by determining their *ob* genotype; and
- (b) selecting animals that possess the T-containing allele of the *ob* gene for inclusion in the group.
9. The method of claim 8 wherein increasing milk production in a selected group of livestock animals of the same species occurs during the first one hundred days of lactation.
- 15 10. The method of claim 9 wherein determining comprises determining whether the animal is a TT animal homozygous with respect to the T-allele of the *ob* gene, a CC animal homozygous with respect to the C-allele of the *ob* gene, or a CT animal heterozygous with respect to the T-allele and the C-allele of the *ob* gene.
- 20 11. A method of claim 10 wherein selecting is selecting from the group consisting of TT animals homozygous with respect to the T-allele of the *ob* gene and CT animals heterozygous with respect to the T-allele and the C-allele of the *ob* gene.
- 25 12. A method of identifying those animals having increased milk productivity compared to general population of animals of the same species by determining their *ob* genotype wherein animals that possess the T-containing allele of the *ob* gene have increased milk productivity compared to animals that possess only the C-containing allele of the *ob* gene.
13. A method of claim 12 wherein TT animals homozygous with respect to the T-allele of the *ob* gene have a greater milk productivity than CT animals heterozygous with respect to the T-allele.
- 30 14. A method of breeding livestock animals to increase milk production in the offspring comprising selecting breeding pairs of livestock animals of the same species to increase occurrence of the *ob* T-allele in the offspring.

15. The method of claim 14 wherein the milk production is increased in the first one hundred days of lactation in the offspring.

16. A method of increasing milk production in a selected group of livestock animals of the same species comprising:

- 5 (a) determining a genetic predisposition of each animal to produce milk by determining their *ob* genotype;
- (d) selecting animals that possess the T-containing allele of the *ob* gene for inclusion in the group; and
- (e) increasing the amount of feed for in the selected group.

10 17. The method of claim 16 wherein increasing milk production in a selected group of livestock animals of the same species occurs during the first one hundred days of lactation.

15 18. The method of claim 17 wherein determining comprises determining whether the animal is a TT animal homozygous with respect to the T-allele of the *ob* gene, a CC animal homozygous with respect to the C-allele of the *ob* gene, or a CT animal heterozygous with respect to the T-allele and the C-allele of the *ob* gene.

19. A method of claim 18 wherein selecting is selecting from the group consisting of TT animals homozygous with respect to the T-allele of the *ob* gene and CT animals heterozygous with respect to the T-allele and the C-allele of the *ob* gene.

20 20. The method of any one of claims 8, 12, 14 or 18 wherein the livestock animal is a bovine, an ovine, an avian or a swine.

21. The method of claim 20 wherein the livestock animal is a bovine.

22. The method of claim 21 wherein the bovine is a dairy cattle.

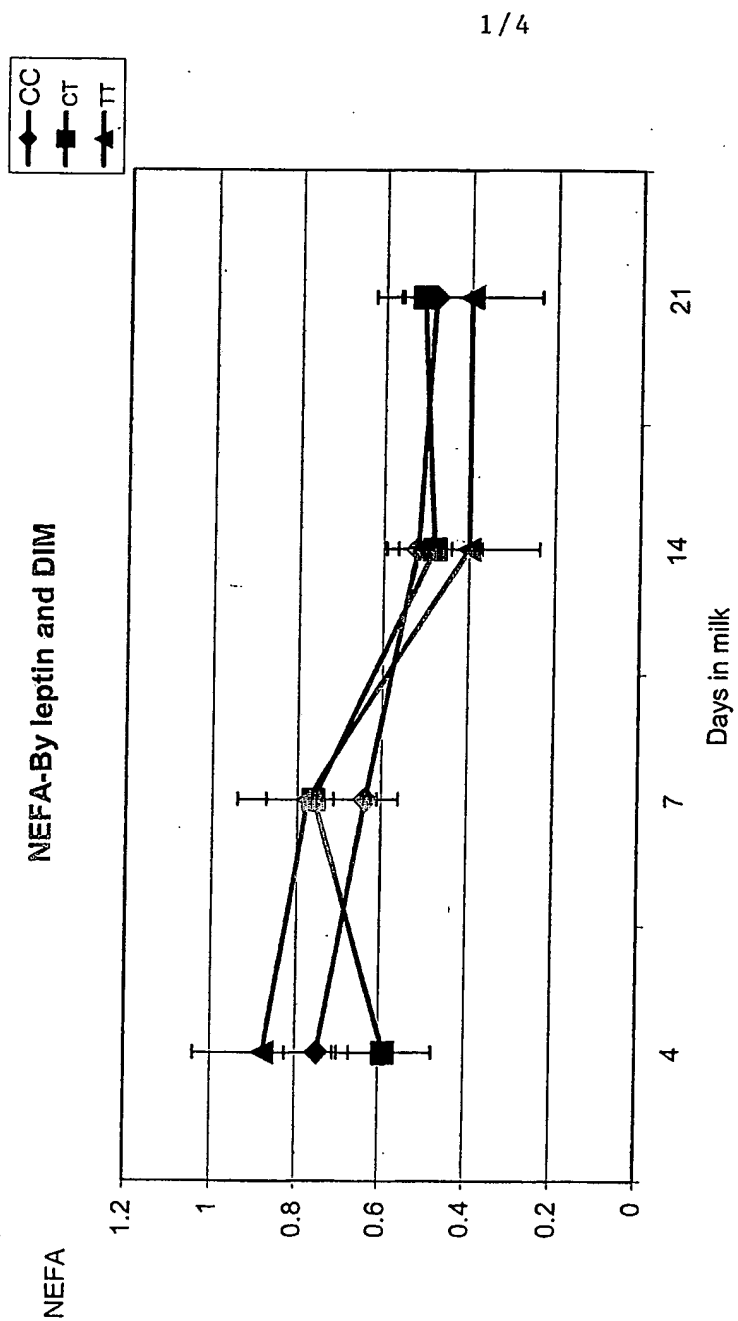


FIG. 1

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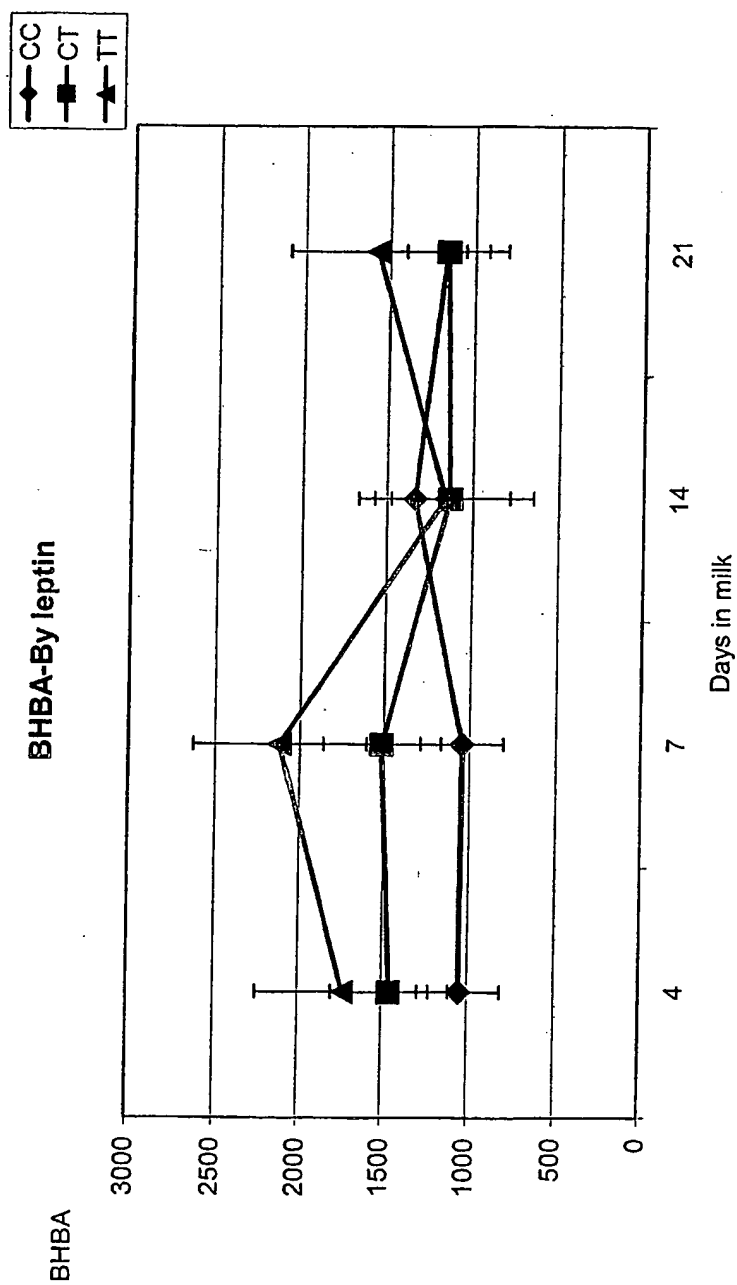


FIG. 2

3/4

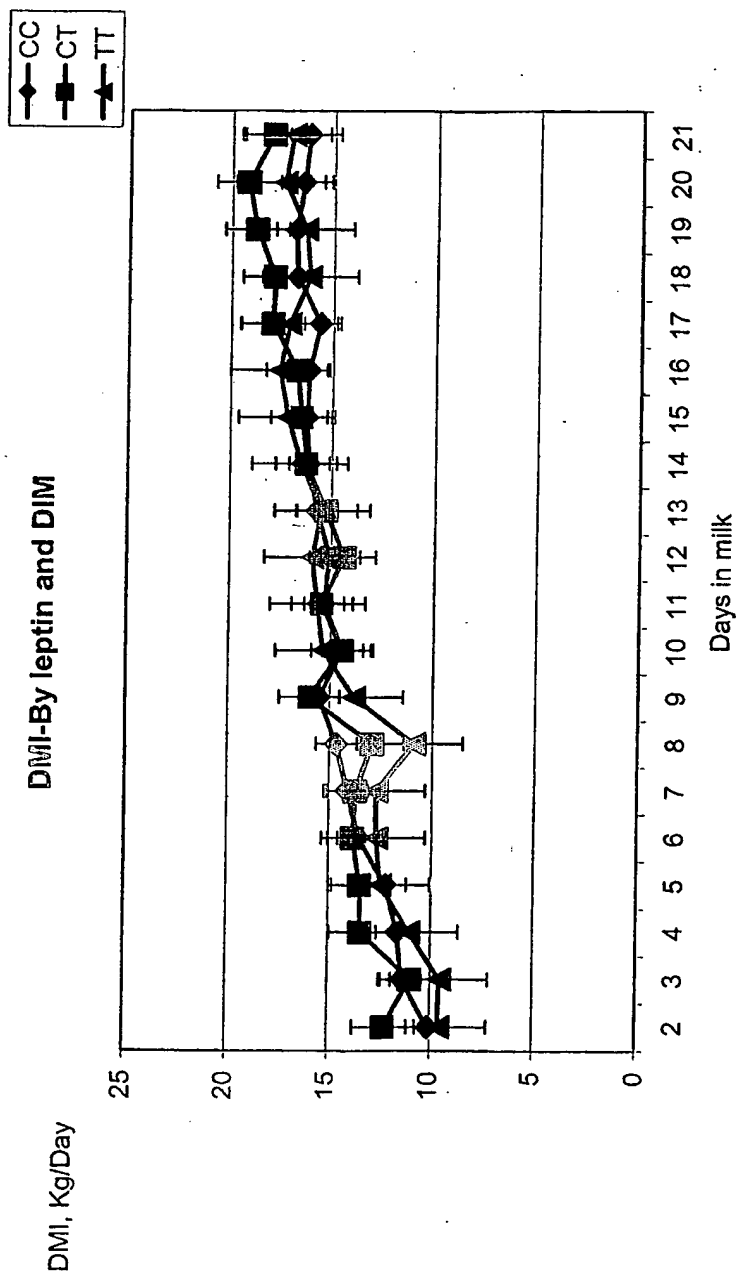


FIG. 3

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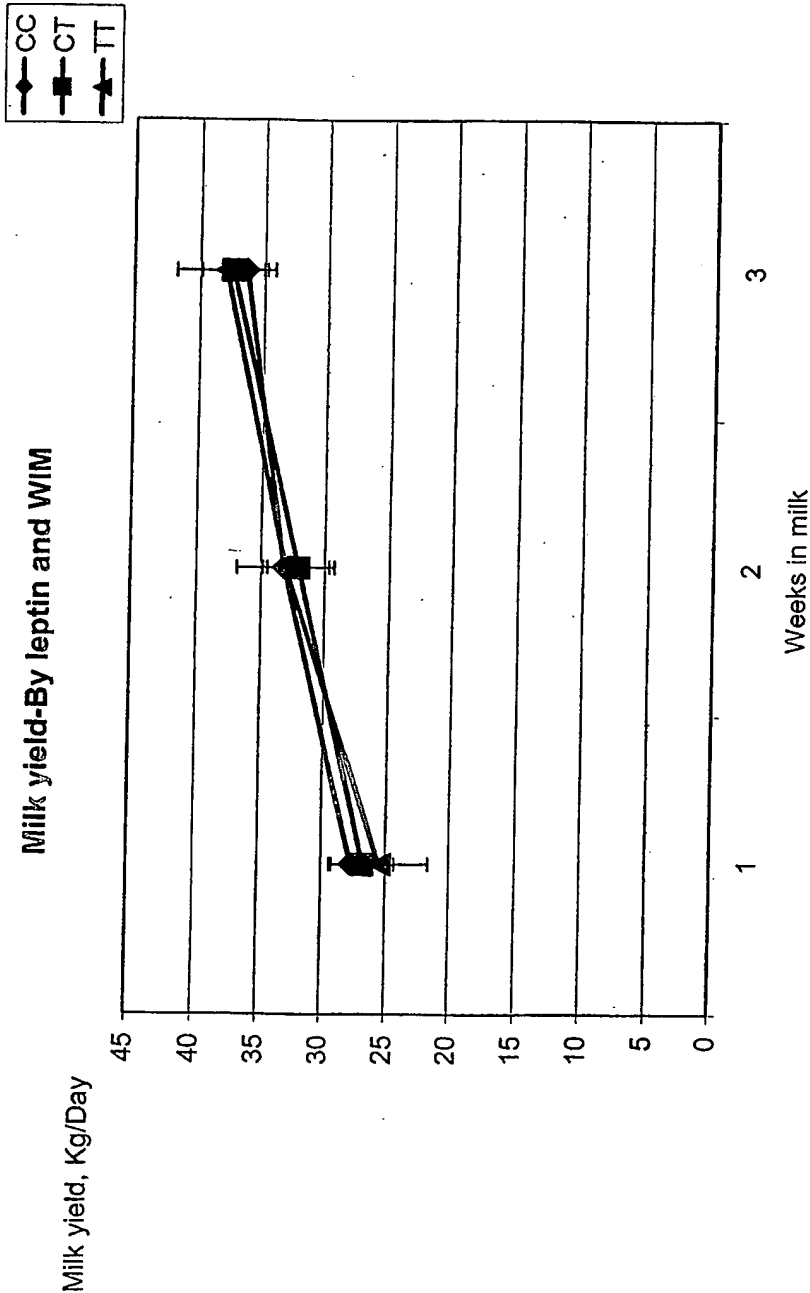


FIG. 4

SEQUENCE LISTING

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INTERNATIONAL SEARCH REPORT

International Application No

T/CA2004/000405

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, MEDLINE, EMBASE, BIOSIS, SEQUENCE SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZWIERZCHOWSKI L ET AL: "AN ASSOCIATION OF GROWTH HORMONE, KAPPA-CASEIN, BETA-LACTOGLOBULIN, LEPTIN AND PIT-1 LOCI POLYMORPHISM WITH GROWTH RATE AND CARCASS TRAITS IN BEEF CATTLE" ANIMAL SCIENCE PAPERS AND REPORTS, POLISH SCIENTIFIC PUBLISHERS, WARZSAW, PL, vol. 19, no. 1, 2001, pages 65-77, XP001127452 ISSN: 0860-4037 page 66 -page 68 page 73 -page 74 --- -/--	1-22

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the International filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the International filing date but later than the priority date claimed

- *T* later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *G* document member of the same patent family

Date of the actual completion of the international search

29 June 2004

Date of mailing of the international search report

05/07/2004

Name and mailing address of the ISA

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Fax (+31-70) 340-3016

Authorized officer

BROCHADO GARGANTA, M

INTERNATIONAL SEARCH REPORT

International Application No
T/CA2004/000405

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>BUCHANAN F C ET AL: "ASSOCIATION OF A MISSENSE MUTATION IN THE BOVINE LEPTIN GENE WITH CARCASS FAT CONTENT AND LEPTIN MRNA LEVELS"</p> <p>GENETICS SELECTION EVOLUTION, EDP SCIENCES, LES ULIS,, FR, vol. 34, no. 1, January 2002 (2002-01), pages 105-116, XP008023609 ISSN: 1297-9686 page 105 -page 108 page 111 -page 113</p>	1-22
A	<p>AOKI N ET AL: "Lactation-dependent down regulation of leptin production in mouse mammary gland"</p> <p>BBA - GENERAL SUBJECTS, ELSEVIER SCIENCE PUBLISHERS, NL, vol. 1427, no. 2, 19 April 1999 (1999-04-19), pages 298-306, XP004276309 ISSN: 0304-4165 page 298 -page 300 page 302 -page 305</p>	1-22
A	<p>RICHARDS M-P ET AL: "Analysis of leptin gene expression in chickens using reverse transcription polymerase chain reaction and capillary electrophoresis with laser-induced fluorescence detection"</p> <p>JOURNAL OF CHROMATOGRAPHY A, ELSEVIER SCIENCE, NL, vol. 853, no. 1-2, 20 August 1999 (1999-08-20), pages 321-335, XP004178251 ISSN: 0021-9673 the whole document</p>	1-22
A	<p>KLAUZINSKA M ET AL: "COMPARISON OF SELECTED GENE POLYMORPHISMS IN POLISH RED AND POLISH BLACK-AND-WHITE CATTLE"</p> <p>ANIMAL SCIENCE PAPERS AND REPORTS, POLISH SCIENTIFIC PUBLISHERS, WARZSAW, PL, vol. 18, no. 2, 2000, pages 107-116, XP001127451 ISSN: 0860-4037 the whole document</p>	1-22
A	<p>WO 02/20850 A (MALEK MASSOUD ;PLASTOW GRAHAM (US); CIOBANU DANIEL C (US); IOWA ST) 14 March 2002 (2002-03-14) the whole document</p>	1-22

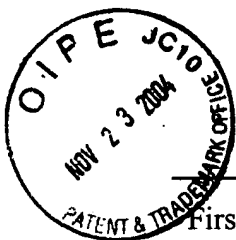
INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

T/CA2004/000405

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 0220850	A	14-03-2002	AU 8898001 A	22-03-2002
			CA 2421754 A1	14-03-2002
			CN 1483081 T	17-03-2004
			EP 1354061 A2	22-10-2003
			WO 0220850 A2	14-03-2002
			US 2003017470 A1	23-01-2003



First Named

Inventor : Michael E. Spurlock

Appln. No. : 09/932,888

Filed : August 20, 2001

Title : Porcine Leptin Protein, Antisense and Antibody

Docket No. : LL31.12-0016

Group Art Unit: 1647

Examiner: C. J. Saoud

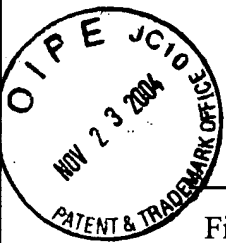
EXHIBIT J

of

DECLARATION

submitted under 37 C.F.R. 1.132

US Patent No. 6,777,388



First Named Inventor : Michael E. Spurlock Appln. No. : 09/932,888 Filed : August 20, 2001 Title : Porcine Leptin Protein, Antisense and Antibody Docket No. : LL31.12-0016	Group Art Unit: 1647 Examiner: C. J. Saoud
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EXHIBIT K

of

DECLARATION

submitted under 37 C.F.R. 1.132

Connolly, Amy L. and Jones, Teri L., Hybridization Buffers I: Comparison of Formamide vs. Aqueous Hybridization Solutions, four pages (KPL Research & Development - August, 2002)

Hybridization Buffers I: Comparison of Formamide vs. Aqueous Hybridization Solutions

By Amy L. Connolly, M.S., and Teri L. Jones, Ph.D.
KPL Research & Development

KPL offers the flexibility of two types of hybridization buffers for use in Northern and Southern blot detection, one a formamide-based solution and the second an aqueous buffer. Formamide Hybridization Buffer (Cat. No. 50-86-10) has been designed as part of KPL's Non-Radioactive Detector™ Systems to provide optimal performance in nucleic acid blotting applications. A destabilizer, formamide lowers the melting temperature of hybrids thus increasing the stringency of the probe to target binding. Use of this agent with specified hybridization temperatures results in minimal nonspecific hybridization; less optimization of washes is required by the end user. Formamide Hybridization Buffer is a suitable universal hybridization solution.

For those researchers wishing to minimize hazardous waste, the aqueous Membrane Hybridization Buffer (Cat. No. 50-86-08) is a non-hazardous alternative. It is not recommended for all hybridization applications as will be shown in this paper. However, Membrane Hybridization Buffer does perform very well for hybridizations with plasmid DNA and moderately expressed transcripts.

To demonstrate the utility of the Formamide and Membrane Hybridization Buffers, a series of comparative studies were conducted and summarized in this Application Note. Both buffers were tested for relative performance in Northern and Southern blots, detecting plasmid DNA as well as low and abundantly expressed transcripts.

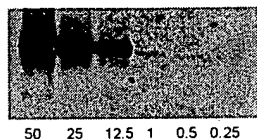
Formamide Hybridization vs. Membrane Hybridization Buffer – Northern Blot Analysis

Using an RNA probe for the detection of a moderate to abundantly expressed message

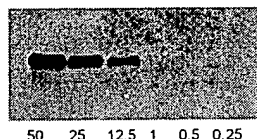
Two-fold serial dilutions starting at 50 ng of total RNA were electrophoresed on a 1% formaldehyde gel. Using alkaline transfer, the RNA was blotted to Biotinyne® B membrane. Hybridization and detection were as follows:

1. The membrane was cut in half to represent two duplicate dilution series. One membrane was pre-hybridized for 1 hour at 65°C in Formamide Hybridization Buffer + 100 µg/mL Herring Sperm DNA. The second membrane was pre-hybridized for 30 minutes at 50°C in Membrane Hybridization Buffer + 200 µg/mL Herring Sperm DNA.
2. A biotinylated 18s riboprobe was denatured at 68°C for 10 minutes and immediately placed on ice. (Note: The RNA probes in these studies were generated via *in vitro* transcription using KPL's Detector RNA *in vitro* Transcription Biotinylation Kit, Cat. No. 60-01-02.)
3. The probe was added to each of the blots at a final concentration of 50 ng/mL of hybridization solution.
4. Blots were hybridized overnight at the same temperature as the pre-hybridization step (65°C in Formamide Hybridization Buffer and 50°C in Membrane Hybridization Buffer, respectively).
5. The blot hybridized in the Formamide Hybridization Buffer was washed 2 x 15 minutes at room temperature in 2X SSPE + 0.1% SDS followed by 2 x 30 minute washes at 65°C in 0.2X SSPE + 0.1% SDS and one final wash for 5 minutes in 5X SSPE.
6. The blot hybridized in the Membrane Hybridization Buffer was washed 2 x 10 minutes at room temperature in 0.5X SSPE followed by 2 x 10 minutes at 55°C.
7. Chemiluminescent detection was performed according to the protocol in the Detector™ AP Chemiluminescent Blotting Kit (Cat. No.'s 54-30-01/02) manual. Exposures of the blots to KODAK BIOMAX Light film were performed at 1 and 10 minutes.

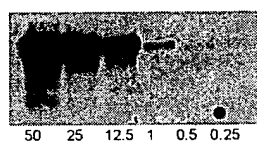
Data and Conclusions



KPL's Formamide Hybridization Buffer
1 minute exposure
(ng RNA)



KPL's Membrane Hybridization Buffer
1 minute exposure
(ng RNA)



KPL's Membrane Hybridization Buffer
10 minute exposure
(ng RNA)

Results of the comparison show that the membranes were sufficiently hybridized such that the riboprobe was able to specifically bind to the target. Additionally, the level of sensitivity achieved is comparable with both hybridization buffers when moderate to abundant messages are probed. The 18s rRNA was detectable to 0.25 ng in both cases. The difference between the blots, however, is observed in the relative sensitivity by exposure time. The Formamide Hybridization Buffer generated stronger signal in a 1-minute exposure to film, whereas equivalent sensitivity was achieved in the blot hybridized with the Membrane Hybridization Buffer after a 10-minute film exposure.

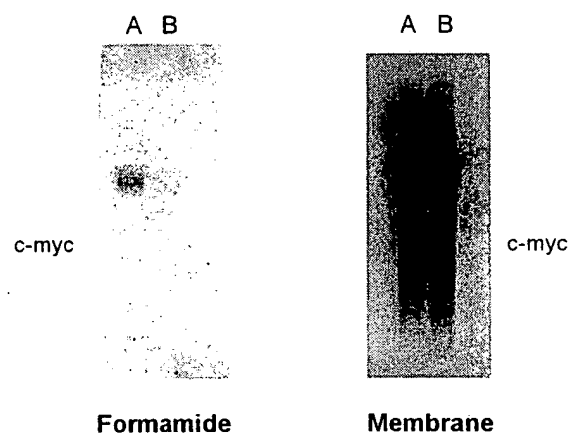
Using an RNA probe for detection of a low expressed transcript

Duplicate lanes of 5 μ g of total RNA from WEH1-231 untreated and anti-IgM treated cells were electrophoresed on a 1% formaldehyde gel. RNA was transferred by a 2 hour alkaline method to Biodyne B membrane. Hybridization and detection were as follows:

1. The membrane was cut in half, each blot containing control and treated RNA. One membrane was pre-hybridized for 1 hour in Formamide Hybridization Buffer + 100 μ g/mL Herring Sperm DNA at 65°C. The second membrane was pre-hybridized for 30 minutes in Membrane Hybridization Buffer + 200 μ g/mL at 55°C.
2. A biotinylated c-myc riboprobe was denatured at 68°C for 10 minutes and immediately placed on ice.

3. Denatured probe was added to each of the blots to a final concentration of 50 ng/mL of hybridization solution.
4. Blots were hybridized overnight at the same temperature as the pre-hybridization step (68°C in Formamide Hybridization Buffer and 55°C in Membrane Hybridization Buffer, respectively).
5. The blot hybridized in the Formamide Hybridization Buffer was stringently washed 2 x 15 minutes at room temperature in 2X SSPE + 0.5% SDS followed by 2 x 30 minutes at 68°C in 0.2X SSPE + 0.5% SDS and one final wash for 5 minutes in 5X SSPE.
6. The blot hybridized in the Membrane Hybridization Buffer was stringently washed 2 x 10 minutes at room temperature in 0.5X SSPE followed by 2 x 10 minutes at 55°C.
7. Using the Detector™ AP Chemiluminescent Blotting Kit, detection was carried out by standard protocol utilizing 0.5% Detector Block Powder in the block solution. The membrane was exposed to KODAK BIOMAX Light film for 1 minute following a 1 hour incubation.

Data and Conclusions



According to the design of this assay, c-myc mRNA should be observed as a single band in the control sample (A) and appear down regulated in the treated sample (B). The blot hybridized with Formamide Hybridization Buffer delivers the expected result. RNA-RNA hybrids are the most stable of the nucleic acid hybrids and thus the most difficult to disassociate even when they are not completely complementary. In this study, Formamide Hybridization Buffer effectively minimized the presence of non-specific hybrids.

However, detection of the transcript with the Membrane Hybridization Buffer could not be achieved.

Significant non-specific binding of the probe to the total RNA was seen when using the Membrane Hybridization Buffer. Multiple attempts were made to increase the stringency of both the hybridization temperature and the washes (data not shown); each blot produced the same pattern. Although the Membrane Hybridization Buffer worked well for the abundantly expressed message, the use of the aqueous Membrane Hybridization Buffer is not recommended in the detection of a low expressed message with a riboprobe. It is also important to note that the stringency of the hybridization temperature and the post-hybridization washes may need to be optimized for each expressed mRNA as detailed in the two experiments shown thus far.

Formamide Hybridization Buffer vs. Membrane Hybridization Buffer – Southern Blot Analysis

Using a random primed DNA probe to detect plasmid DNA

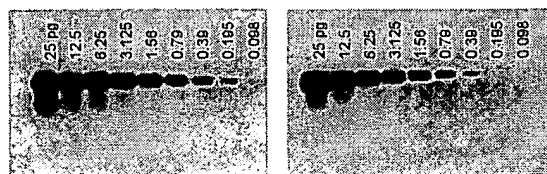
Two-fold serial dilutions of plasmid DNA containing a timp-2 insert were prepared, starting with 25 pg of insert. The dilution series was loaded in duplicate on an agarose gel and subsequently electrophoresed, denatured and neutralized by standard methods. The gel was transferred overnight onto Biotrans® B, a positively charged nylon membrane.

1. The membrane was cut in half to represent two duplicate dilution series. One membrane was pre-hybridized for 1 hour in Formamide Hybridization Buffer + 100 µg/mL Herring Sperm DNA at 42°C. The second membrane was prehybridized for 30 minutes in Membrane Hybridization Buffer + 200 µg/mL at 50°C.
2. A timp-2 random primed biotinylated DNA probe was denatured at 95°C for 10 minutes and immediately placed on ice. (Note: The DNA probe used in this study was biotinylated via KPL's Detector™ Random Primer DNA Biotinylation Kit, Cat. No. 60-01-00.)
3. Denatured probe was added to each of the blots to a final concentration of 50 ng/mL of hybridization solution.
4. Blots were hybridized overnight at the same temperature as the pre-hybridization step (42°C in Formamide Hybridization Buffer and 50°C in Membrane Hybridization Buffer, respectively).
5. The blot hybridized with Formamide Hybridization Buffer was stringently washed 2 x 15 minutes at room temperature in 2X SSPE + 0.1% SDS followed by 2 x 30 minutes at 65°C in 0.2X SSPE + 0.1% SDS and one final wash for 5

minutes in 5X SSPE.

6. The blot hybridized in the Membrane Hybridization Buffer was stringently washed 2 x 10 minutes at room temperature in 0.5X SSPE followed by 2 x 10 minutes at 50°C.
7. Detection was carried out by standard protocol using the Detector™ HRP Chemiluminescent Blotting Kit (Cat. No. 54-30-00). Exposure to KODAK BIOMAX Light film was performed for 10 minutes.

Data and Conclusions



Formamide

Membrane

Both hybridization buffers worked equally well in the detection of timp-2, each resulting in the detection of as low as 98 fg of plasmid DNA. Relatively, the blot hybridized with the Formamide Hybridization Buffer yielded slightly higher sensitivity with the same exposure time to film. For this type of hybridization where the complexity of the target is not an issue, either buffer is suitable when used under the recommended standard stringent conditions.

However, as the results in the hybridization and detection of the low expressed mRNA show, a similar phenomenon is found in the use of Membrane Hybridization Buffer in single copy genomic Southern blots. As the amount of target in the total sample becomes proportionately smaller, the requirements for stringency become greater and the need for further optimization of this hybridization solution also increases. Likewise, greater stringency is required for the hybridization to allow for the specific detection of single copy genes. Therefore, Membrane Hybridization Buffer is not recommended for this application.

Related products:

Description	Size	Catalog No.
Formamide Hybridization Buffer	240 mL	50-86-10
Membrane Hybridization Buffer	240 mL	50-86-08
Detector™ HRP Chemiluminescent Botting Kit	2000 cm ²	54-30-00
Detector™ AP Chemiluminescent Blotting Kit	2000 cm ²	54-30-01
	500 cm ²	54-30-02
Detector™ Random Primer DNA Biotinylation Kit	30 reactions	60-01-00
Detector™ PCR DNA Biotinylation Kit	30 reactions	60-01-01
Detector™ RNA <i>in vitro</i> Transcription Biotinylation Kit	20 reactions	60-01-02
GeneRuler™ Biotinylated DNA Ladder	20 - 50 lanes	600-0008
Herring Sperm DNA	40 mg	60-00-14
Biodyne® B Nylon Membrane	20 cm x 1 mL roll	60-00-50
Hybridization Bags, 8" x 10"	50/pk	60-00-51

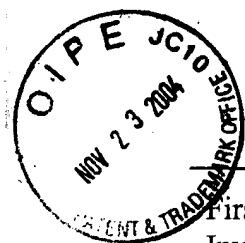
GeneRuler™ is a trademark of Fermentas.

Biodyne® is a registered trademark of Pall Corporation.



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ML-275-01



First Named

Inventor : Michael E. Spurlock

Appln. No. : 09/932,888

Filed : August 20, 2001

Title : Porcine Leptin Protein, Antisense and Antibody

Docket No. : LL31.12-0016

Group Art Unit: 1647

Examiner: C. J. Saoud

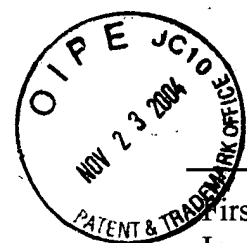
EXHIBIT L

of

DECLARATION

submitted under 37 C.F.R. 1.132

US20040137492A1



First Named

Inventor : Michael E. Spurlock

Appln. No. : 09/932,888

Filed : August 20, 2001

Title : Porcine Leptin Protein, Antisense and Antibody

Docket No. : LL31.12-0016

Group Art Unit: 1647

Examiner: C. J. Saoud

EXHIBIT M

of

DECLARATION

submitted under 37 C.F.R. 1.132

Selmi-Ruby, Samia; Watrin, Chantal; Trouttet-Masson, Severine; Bernier-Valentin, Françoise; Flachon, Virginie; Munari-Silem, Yvonne; and Rousset, Bernard; The Porcine Sodium/Iodide Symporter Gene Exhibits an Uncommon Expression Pattern Related to the Use of Alternative Splice Sites not Present in the Human or Murine Species;
Endocrinology Vol. 144, No. 3, pages 1074-1085 (2003)

The Porcine Sodium/Iodide Symporter Gene Exhibits an Uncommon Expression Pattern Related to the Use of Alternative Splice Sites not Present in the Human or Murine Species

SAMIA SELMI-RUBY, CHANTAL WATRIN, SEVERINE TROUTTET-MASSON, FRANÇOISE BERNIER-VALENTIN, VIRGINIE FLACHON, YVONNE MUNARI-SILEM, AND BERNARD ROUSSET

Institut National de la Santé et de la Recherche Médicale, Unité 369, Institut Fédératif de Recherche Laennec, 69372 Lyon, Cedex 08, France

The sodium/iodide symporter (NIS) is a membrane protein mediating the active transport of iodide into the thyroid gland. NIS, expressed by human, rat, and mouse thyrocytes, is encoded by a single transcript. We identified NIS mRNA species of 3.5 and 3 kb in porcine thyrocytes. Because porcine thyrocytes in primary culture is a widely used experimental system for thyroid iodide metabolism, we further examined the origin and the function of the porcine NIS (pNIS) transcripts. We generated a porcine thyroid cDNA library from which four different clones, pNIS-D, F, J, and Δ J were isolated. pNIS-D encodes a protein of 643 amino acids highly homologous to the human, rat, and mouse NIS. pNIS-F and J differ from each other and from pNIS-D in their C-terminal part. pNIS- Δ J lacks a six-amino-acid segment within the putative transmembrane domain 10. Transiently expressed in Cos-7 cells, the four pNIS-cDNAs led to the synthesis of proteins

targeted at the plasma membrane and conferred perchlorate-sensitive iodide uptake activities to Cos-7 cells, except pNIS- Δ J, which was devoid of activity. pNIS-D probably derives from the 3.5-kb transcript and pNIS-F, J, and Δ J from the 3-kb transcript. The relative abundance of pNIS-D, F, and J transcripts in porcine thyrocytes was about 60%, 35%, and 5%, respectively; the Δ J transcript was not present in detectable amount. By comparing porcine NIS genomic and cDNA sequences, splice donor and acceptor sites accounting for the generation of pNIS-F, J, and Δ J transcripts were identified. None of the combinations of alternative splice sites found in the pig was present in the human, rat or mouse NIS gene. Our data show that porcine NIS gene, contrary to the NIS gene from other species, gives rise to splice variants leading to three active and one inactive NIS proteins. (*Endocrinology* 144: 1074–1085, 2003)

THE ACTIVE TRANSPORT of iodide into the thyroid gland is the first and a rate-limiting step in the biosynthesis of thyroid hormones. Iodide transport is mediated by the sodium/iodide symporter (NIS) located at the basolateral plasma membrane of thyroid follicular cells (recently reviewed in Refs. 1 and 2). NIS cotransports two sodium ions along with one iodide ion, with the transmembrane sodium gradient serving as the driving force for iodide uptake. The sodium gradient providing the energy for this transfer is generated by the Na^+/K^+ ATPase. NIS functional activity is blocked by the competitive inhibitor, perchlorate. It has long been known that TSH, which is the main thyroid regulator, controls iodide uptake (3). If the biochemical and physiological properties of NIS have been known since several decades, its amino acid sequence was more recently determined by expression cloning in *Xenopus laevis* oocytes from a cDNA library derived from rat thyroid cells, the FRTL-5 cell line (4). Using the sequence information of rat NIS (rNIS), the human homolog (hNIS) was cloned soon after (5) and more recently the mouse NIS (mNIS) was identified (6, 7). As several tissues other than thyroid are also capable of trapping

iodide, different investigators compared NIS sequence expressed in rat thyroid and rat extrathyroidal tissues such as salivary glands (8), gastric mucosa (9), and mammary glands (6). Except for a few nucleotide substitutions, the same rNIS sequences were found; this finding suggests that the NIS protein expressed in the thyroid and extrathyroidal tissues derived from the same gene. The human NIS gene, mapped to chromosome 19 (19p12.13.2), consists of 15 exons extending on more than 20 kb (10). In all species studied so far, NIS is encoded by a single transcript, the size of which varies from 2.9 kb in rats to 3.7 kb in humans, giving rise to a 80- to 90-kDa glycosylated protein predicted to possess 13 transmembrane domains (11).

One of the privileged *in vitro* models to analyze different aspects of thyroid cell biology, especially iodine metabolism and thyroid hormone biosynthesis, is the porcine thyroid cell primary culture system. Indeed, freshly isolated pig thyrocytes are capable of reconstituting functional thyroid follicles in short-term culture (12–14), allowing one to study the expression of thyroid differentiation *in vitro*, under conditions rather close to the *in vivo* physiological context. With the aim of analyzing the regulation of NIS expression in this model, we tried to identify porcine NIS (pNIS) by Western blot, using antipeptide antibodies recognizing the C-terminal domain of either rNIS (15) or hNIS (Trouttet-Masson S., S.

Abbreviations: AG, Alternative splice acceptor site; hNIS, human NIS; IUA, iodide uptake activity; mNIS, mouse NIS; NIS, sodium/iodide symporter; pNIS, porcine NIS; rNIS, rat NIS; SDS, sodium dodecyl sulfate.

Selmi-Ruby, F. Bernier-Valentin, and B. Rousset, unpublished data). None of the antibodies did react with any porcine thyroid molecular species. Similarly, cDNA probes used to detect the rNIS transcript by Northern blot were ineffective for the identification of NIS transcripts in pig. Using a cDNA fragment generated by PCR with primers corresponding to conserved sequences in rNIS and hNIS, we found that pig thyrocytes contained two NIS transcripts of about 3.5 and 3 kb. This preliminary information suggesting substantial species differences in NIS primary structure and expressed forms prompted us to generate a porcine thyroid cDNA library to clone pNIS. Here, we report that the pNIS gene gives rise to different transcripts that are produced by the use of alternative splice sites not present in the NIS gene from other species (human, rat, and mouse). Among pNIS transcripts, three encode a functional protein and the fourth one, exhibiting a deletion in a putative transmembrane domain number 10, encodes a protein targeted to the plasma membrane but without activity.

Materials and Methods

Thyroid cell culture

Thyroid cells were dispersed from thyroid glands of adult pigs according to Ref. 16. Freshly dispersed thyrocytes were cultured in Petri dishes ($\varnothing = 10$ cm) in Ham's F12 medium (Seromed Biochrom KG, Berlin, Germany) containing penicillin (200 U/ml), streptomycin (200 μ g/ml) and amphotericin-B (0.5 μ g/ml), and 10% calf serum (Life Technologies, Inc. SARL, Cergy Pontoise, France) at 37 C under air/CO₂ (95%/5%) atmosphere. Cells cultured at a density of 0.5×10^6 cells/cm², in the presence of TSH (1 mU/ml) from the time of seeding, reorganized into histiotypic structures named reconstituted thyroid follicles (14, 17), whereas, in the absence of TSH, they formed but a monolayer.

RNA extraction

Total RNA was extracted from thyrocytes or Cos-7 transfected cells according to the phenol-chloroform method of Chomczynski and Sacchi (18). Purification of mRNA was carried out using a kit from Promega Corp. (Madison, WI). The RNA concentration was determined by absorbance measurements at 260 nm and the RNA purity and integrity were assessed by determination of the A260/A280 ratio and ethidium bromide staining of ribosomal 28S and 18S bands after electrophoretic separation on 1% agarose gels.

Molecular cloning of pNIS from a pig thyroid cDNA library

A cDNA library was synthesized from poly A⁺ RNA prepared from porcine thyroid cells cultured for 4 d in the presence of TSH, using a cDNA synthesis kit and the XhoI-oligo-deoxythymidine primer (Origene Technologies, Inc., Rockville, MD). An EcoRI adapter was ligated at the 5' end of cDNAs that were then cloned into the EcoRI-SalI sites of the pCMV6-XL3 vector owing to the fact that XhoI and SalI have compatible cohesive ends. The pig cDNA library containing about 5.4×10^6 independent clones was screened by hybridization according to standard procedures (19) using a 0.8-kb porcine NIS cDNA fragment generated by RT-PCR (probe A). The primers used to generate probe A derived from the rat NIS (rNIS) sequence; they corresponded to nucleotides 559–584: 5'CGC GCC TGC GCT CAT CCT GAA CCA AG3' and to nucleotides 1309–1333: 5'CAG CAG TGA GGA CAG AGC CAC AG3' of the rNIS cDNA coding region (4). The amplified cDNA was cloned into the pGEM-T vector and certified by sequencing (Genome Express, Grenoble, France) using templates from the pGEM-T vector. Finally, the probe generated by digestion with SphI and PstI was randomly labeled with [α^{32} P]-deoxy-CTP. After overnight hybridization at 42 C with the labeled probe, nylon membranes were washed at high stringency at 65 C in 0.2 \times SSC, 0.5% sodium dodecyl sulfate (SDS) (1 \times SSC corresponds

to 150 mM NaCl and 15 mM sodium citrate at pH 7.0); positives clones were selected and amplified.

Preparation of pNIS cDNA constructs

Constructs corresponding to each pNIS cDNA clone isolated from the porcine thyroid cDNA library were generated by PCR. To optimize expression, the 5' and 3' untranslated regions of pNIS cDNAs were removed. PCR amplification was performed with 200 ng of cDNA template, 50 pmol of each primer in 5 μ l 10 \times reaction buffer, and 2.6 U Expand High Fidelity Taq polymerase (Roche Diagnostics, Meylan, France) in a final volume of 50 μ l. As the 5' end of the open reading frame was the same in all clones, we used the same forward primer to generate the different constructs. The primer sequence corresponding to nucleotides (1–30), numbered in the 5' to 3' orientation and beginning with the first base of the start codon, was: 5'ATG GCG ACC GTC GAG GGA GGC GCG CGG GCC3'. The reverse primer represented the complementary sequence of the C terminus of each pNIS cDNA clone followed by a BamHI site (GAGGGATCC); the sequence of the primers was: 5'CGC TTA GAG GTC CGT CTC ACG CAG GTC3' for pNIS-D (1908–1934); 5'TCA GCA TCC ACC TTT GTC ATG TTC ACT GC3' for pNIS-F (1884–1912) and 5'GAG ATG CAT AAA GTG TCT AGA CGC TGA AG3' for pNIS-J (2036–2064) and pNIS-AJ (2019–2047). Other constructs containing pNIS cDNA sequences fused in 5' to the sequence encoding the Flag epitope were generated by PCR using an oligonucleotide with a start codon followed by the 21 nucleotides corresponding to the Flag sequence and the first 24 bases of the 5' end of pNIS coding region. The 3' primers were those described above. The amplification reaction included a presoak step at 94 C for 3 min followed by 35 cycles for 1 min at 94 C, 1 min at 65 C, and 1 min at 72 C followed by a final expansion period of 10 min at 72 C. PCR products were cloned into the pTarget vector (Promega Corp.). The sequence of each construct, either untagged or N-Flag tagged, was verified by automated sequencing.

Transient transfection of Cos-7 cells

Cos-7 cells were maintained and propagated in DMEM containing 10% (vol/vol) fetal calf serum, at 37 C under air/CO₂ (95%/5%) atmosphere. One day before transfection, cells were trypsinized and plated on 10-cm culture dishes. Cells at about 80% of confluency were transfected with pNIS cDNA vectors using Eugene transfection reagent (Roche Diagnostics; 0.7 μ l of Eugene 6/ μ g DNA) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were assayed for NIS expression by Northern blot, Western blot, and indirect immunofluorescence using anti-Flag antibodies and for iodide uptake activity.

Western blot analysis

Membrane fractions from NIS expressing Cos-7 cells were prepared as described in Ref. 20. In brief, transfected cells were washed with PBS, harvested, and resuspended in PBS containing aprotinin, leupeptin, and pepstatin (each at a concentration of 1 μ g/ml), and 1 mM phenyl methylsulfonyl fluoride. Cells were then lysed by a freezing/thawing cycle and sonicated for 40 sec at 25 W using the vibra-cell apparatus from Bioblock Scientific (Illkirch, France). Crude membrane fractions were obtained by centrifugation at 100,000 \times g for 20 min at 4 C. Protein concentration was assayed by the Lowry method after solubilization in 0.1% sodium desoxycholate using BSA as standard. Membrane protein samples (40 μ g) were fractionated by electrophoresis on 9% polyacrylamide gel in presence of SDS and electrotransferred onto Immobilon-P membranes (Millipore Corp., Molsheim, France). Immobilon-P membranes were preincubated with 5% (wt/vol) nonfat dry milk and 0.2% (vol/vol) Tween-20 in PBS for 1 h at room temperature and incubated with a mouse anti-Flag M2 monoclonal antibody (Sigma-Aldrich, St. Quentin Fallavier, France) at a 1:2000 final dilution, overnight at 4 C. After three washes in PBS-0.2% Tween solution, Immobilon-P membranes were incubated with an antimouse IgG conjugated to horseradish peroxidase (Bio-Rad Laboratories, Inc., Richmond, CA; 1:5000 final dilution) for 1 h at room temperature. Detection of immune complexes was performed using an enhanced chemiluminescence kit from Amersham (Orsay, France) followed by an exposure for 1–3 min to Kodak (Rochester, NY) X-OMAT-AR film.

Indirect immunofluorescence

Cos-7 cells were fixed with 4% paraformaldehyde for 30 min at room temperature, then washed three times with PBS containing 10 mM glycine. Cells were permeabilized or not with 1% Triton X-100 in the same buffer for 10 min and washed three times with PBS containing 10 mM glycine. Cells were then preincubated in PBS containing 4% BSA for 1 h and incubated with the mouse anti-Flag M2 monoclonal antibody at a 1:2000 dilution in PBS containing 4% BSA for 1 h. Cells were washed and further incubated with a fluorescein-labeled goat antimouse secondary antibody (1:100 dilution; DAKO Corp., Trappes, France) for 1 h in the dark. Nuclei were stained with 1 μ g/ml Hoechst-33342 reagent (Molecular Probes, Eugene, OR) for 10 min at room temperature. Fluorescence images, obtained with a Carl Zeiss (Jena, Germany) Axiophot fluorescence microscope, were monitored using a cooled charge-coupled device camera (LHESA Electronique, Cergy Pontoise, France). Images were collected in a computer equipped with a Cyclope imaging card (Digital Vision, Chatillon, France) and analyzed using graphic software.

Iodide uptake activity (IUA) measurements

Transfected cells were trypsinized, distributed into 3-cm wells, and cultured for 24 h. Cells were washed twice with Earle's balanced salt solution, pH 6.8, and incubated in the same medium containing 0.5–1.0 μ Ci Na^{125}I for 40 min at 37°C. Incubations were performed in the absence and in the presence of 0.1 mM sodium perchlorate, a competitive inhibitor of the sodium/iodide symporter. At the end of the incubation period, the medium was removed, and cells were quickly washed twice with ice-cold Earle's medium. The procedure was achieved within 40 sec. Cells were lysed in 200 μ l cell culture lysis buffer (Promega Corp., Madison, WI) for 5 min and the cell lysate was counted in a γ counter from Packard Instruments Co., Inc. (Groningen, The Netherlands). Incubations were made in triplicate.

Analyses of the relative expression of each pNIS splice variant by thyroid cells in primary culture

Total RNA (25 μ g) from cultured thyroid cells was fractionated by electrophoresis on 1% agarose gel containing formaldehyde, transferred to Hybond-N⁺ membrane (Amersham) and hybridized with two different probes (probe A and B) using standard Northern blot procedures (19). Probe A was the 0.8 kb pNIS cDNA fragment described above and probe B was a 476-bp pNIS cDNA fragment generated by PCR using pNIS-D as template and the following primers: 5'-primer (1908–1932) 5'-ACC TGC GTG AGA CGG ACC TCT AA³; 3'-primer (2361–2383) 5'-GTA TAG GGG TTG GGC CTC AGG AC³. Probes were labeled using the random priming kit from Roche Diagnostics. Hybridization was carried out in a 50% (vol/vol) formamide solution containing the [³²P]-labeled probe (1–2 \times 10⁶ cpm/ml), overnight at 42°C. Filters were washed under stringent conditions (0.2 \times SSC, 0.5% SDS at 60°C) and exposed to Kodak XAR-5 films (Eastman Kodak Co.) at –80°C using intensifying screens.

The identification of transcripts corresponding to the different splice variants among pig thyroid cell RNA was pursued using RT-PCR. Two sets of primers, α 1/ α 2 and β 1/ β 2, flanking the alternatively spliced regions were used; their position on the pNIS-D cDNA is shown (see Fig. 5). Their sequence was: α 1(1805–1828) 5'-GAC TTC CTG TCC ACT AAT GAG GAC³; α 2 (2571–2591) 5'-AGT GTC TAG ACG CTG AAG ATG³; β 1 (933–962) 5'-CCT CTC CTG GCA GGG CAT ATC TCT GCC CCA³; and β 2 (1294–1323) 5'-CGT ATT GCA GGA GGG GAG GAA CAT TCC CAG³. One microgram of total RNA isolated from pig thyrocytes was reverse-transcribed with the specific antisense primer and 200 U of RT-SuperScript II (Invitrogen SARL, Cergy Pontoise, France) and used as template for PCR amplification. The protocol included a presoak step at 94°C for 3 min followed by 35 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C followed by a final expansion period of 10 min at 72°C. The resulting PCR products were separated by electrophoresis on 2% agarose gels stained with 0.5 μ g/ml ethidium bromide. The size of the expected fragments generated from α 1/ α 2 primers was 295 and 249 bp for pNIS-F and pNIS-J/ Δ J, respectively. The size of the expected amplicons generated from β 1/ β 2 primers was 393 bp and 377 bp for pNIS-J and pNIS- Δ J, respectively. Given the small difference in size, the am-

plified products were submitted to a BpuI digestion to discriminate between pNIS-J and pNIS- Δ J.

Amplification of genomic DNA

PCR was carried out on 250 ng porcine genomic DNA isolated by the method of Blin and Stafford (21). Amplification of the pNIS gene was carried out with three different sets of primers (A, B, and C). The nucleotide sequence of primers is given by reference to pNIS-D sequence (numbered in the 5' to 3' direction beginning with the first base of the start codon) and exons refer to the hNIS gene structure.

Set A, 5' primer (1275–1301)-exon 8 5'-TGA AGA CCT GCC TGG AGT CCC TGG GCT³;

3' primer (1523–1548)-exon 10 5'-CTG ATG ACT CCC ATG ACG GTG AAT GA³;

Set B, 5' primer (2014–2037)-exon 14 5'-GCT GCC CTG GAT GAC AGC CTG ATG³;

3' primer (2044–2067)-exon 15 5'-TGC CAA AGG CAA TTC CTC AGC ACC³;

Set C, 5' primer (1275–1301)-exon 15 5'-GGT GCT GAG GAA TTG CCT TTG³;

3' primer (2852–2880)-exon 15 5'-GAG ATG CAT AAA GTG TCT AGA CGC TGA AG³.

PCR amplification products were purified using agarose gel electrophoresis and the DNA extraction kit from QIAGEN S.A. (Courtaboeuf, France); they were cloned into the pGEM-T vector and subjected to automated sequencing.

Results

Identification of NIS mRNA in porcine thyrocytes

As our attempts to detect pNIS mRNA by Northern blot, using either rat or human NIS cDNA probes were unsuccessful, we generated a specific pNIS probe by RT-PCR. Based on nucleotide sequence homology between hNIS and rNIS cDNAs (4, 5), primers have been designed and used to amplify an 800-bp product from porcine thyroid total RNA. The nucleotide sequence of the pNIS amplicon showed 81% and 86% identity with rNIS (4) and hNIS (5), respectively. Northern blot analysis of total RNA from porcine thyroid cells using this pNIS cDNA fragment as a probe (probe A) identified two transcripts of about 3.5 and 3 kb (Fig. 1). The two transcripts detected in freshly isolated thyroid cells disappeared within 48 h of culture of thyrocytes in the absence of TSH. When thyrocytes were cultured in the presence of TSH, the level of the two transcripts first declined (at 24 h) then increased up to 5- to 10-fold after 48 or 72 h. Under these various circumstances, the 3.5-kb transcript represented the major form and the relative level of the two transcripts remained rather constant. The identification of two NIS transcripts in pig thyroid gland was in marked contrast with what was known in other species; only one transcript of 3.7

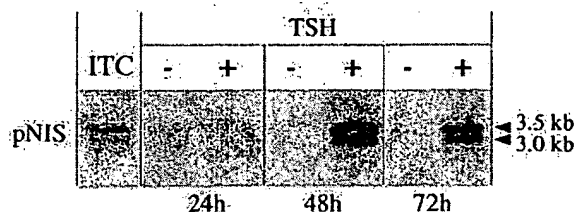


FIG. 1. Identification of pNIS transcripts in porcine thyroid cells. Total RNA was extracted from freshly isolated porcine thyroid cells (ITC) and cells cultured for 24, 48, or 72 h in the absence (–) or in the presence (+) of TSH (1 mU/ml) added at the time of cell seeding. Total RNA (25 μ g) was subjected to Northern blot analysis using probe A. Arrows indicate the size of the pNIS transcripts.

kb had been detected in humans and 2.9 kb in mice and rats, respectively. To determine whether the different pNIS mRNAs encode active Na⁺/I⁻ symporter proteins, we decided to clone pNIS cDNA sequences.

Identification of four different pNIS cDNAs

An oligo (deoxythymidine)-primed porcine thyroid cDNA library (5.4×10^6 independent clones) was generated from purified mRNA extracted from thyrocytes cultured for 4 d in the presence of TSH. This library was screened by hybridization with probe A previously used for the identification of porcine NIS transcripts. Four independent cDNA clones named pNIS-D, pNIS-F, pNIS-J, and pNIS-ΔJ have been isolated. The size of these cDNAs as well as the size of their open reading frame and corresponding polypeptide chain are reported in Table 1. The four cDNA sequences have been submitted and are available in GenBank/EMBL/DDBJ under the accession number mentioned in Table 1. The 3'-untranslated region (variable in length) of the four pNIS cDNAs presented two typical AATAAA polyadenylation signal (22) with a poly (A) tract.

Alignment of amino acid sequences and secondary structure predictions

The ORF of the pNIS-D corresponding to a polypeptide chain of 643 amino acids exhibited 85% identity and 91.5% similarity with the hNIS sequence. The homology with rNIS and mNIS was also very high (79.3% identity and 88% similarity) (Fig. 2). The human NIS and the pNIS-D proteins both composed of 643 amino acids differ from rat and mouse NIS proteins (formed of 618 amino acids) by two insertions of 5 residues in the putative last intracellular loop and 20 residues in the C-terminal intracellular domain. Polypeptide sequences of pNIS-F and pNIS-J, respectively, share the first 635 and 620 amino acids with pNIS-D but present distinct C-terminal sequences (Fig. 2B). The pNIS-ΔJ sequence differs from that of pNIS-J by a six-amino-acid deletion (amino acid 391–396) (Fig. 2C). Secondary structure prediction analysis suggested that pNIS-D (as well as other isoforms) is an intrinsic membrane protein with 13 transmembrane domains with the amino terminal domain located on the extracellular side of the plasma membrane and the carboxy terminal end inside the cytoplasm in accordance with the revised topological analysis of rNIS by Levy *et al.* (11). The three N-glycosylation sites (Asn-X-Ser/Thr) conserved in hNIS, rNIS, and mNIS are present in all pNIS isoforms (positions 225, 489, and 501). The potential phosphorylation site by cAMP-dependent protein kinase at position 554–557 on hNIS, which is conserved in rNIS and mNIS is absent in pNIS isoforms. Two consensus sequences for phosphorylation by

protein kinase A have been found on pNIS at positions 52–55 and 240–243; they are located at the start of predicted α helices forming the putative transmembrane domain nos. 2 and 7.

Functional analysis of pNIS constructs by transient expression in Cos-7 cells

Cos-7 cells were transfected with expression vectors containing each of the four pNIS cDNA in either the untagged or N-Flag-tagged forms. As illustrated on the Western blot of Fig. 3A, the anti-Flag M2 monoclonal antibody recognized a single protein band, with an electrophoretic mobility of approximately 62 kDa, in cells transfected with either pNIS-D, pNIS-F, pNIS-J, or pNIS-ΔJ constructs.

The cellular localization of each pNIS isoform expressed by Cos-7 cells using the anti-Flag antibody is illustrated in Fig. 4. Immunofluorescence images of nonpermeabilized cells revealed the presence of the recombinant proteins at the plasma membrane. A similar labeling was obtained for the four pNIS isoforms. In permeabilized cells (+ Triton X-100), the immunostaining was not restricted to the plasma membrane but was also localized in intracellular compartments.

Cos-7 cells expressing either untagged or N-Flag-tagged pNIS were assayed for their capacity to concentrate iodide. Negative controls were cells transfected with either empty pTarget vector or with vectors containing the different pNIS cDNA in an antisense orientation. The IUA was measured in the absence or in the presence of sodium perchlorate; results are reported in Fig. 3B. Cells transfected with control vectors did not show any IUA. By contrast, a specific perchlorate sensitive iodide uptake was observed in cells expressing pNIS-D, pNIS-F, and pNIS-J. Cos-7 cells expressing the untagged (dark bars) or the N-Flag-tagged pNIS (gray bars) isoforms exhibited the same IUA. Despite a protein expression level and localization indistinguishable from that obtained with the other cDNAs, pNIS-ΔJ did not confer IUA to Cos-7 cells. To demonstrate that the deletion of the 6 residues in the putative transmembrane domain 10 (the difference between pNIS-J and ΔJ) was responsible for the lack of IUA, we generated a pNIS-D construct (pNIS-Δ18) carrying the deletion of 18 nucleotides. No specific iodide transport was observed in cells expressing pNIS-Δ18 (data not shown). Cotransfection of Cos-7 cells with equal amounts of pNIS-D and pNIS-Δ18 or pNIS-J and pNIS-ΔJ resulted in a normal IUA (data not shown), showing that the inactive form of pNIS had no effect on the functional forms.

As shown in Fig. 3C, the three functional pNIS isoforms (pNIS-D, pNIS-F, and pNIS-J) transport iodide with the same K_m value, about 20 μM.

Table 1. Molecular characteristics of the four different porcine NIS (pNIS) cDNA clones isolated from a porcine thyroid cell cDNA library

Cloned cDNA	pNIS-D	pNIS-F	pNIS-J	pNIS-ΔJ	hNIS (1)	rNIS (2)	mNIS (3)
Accession no.	AJ276242	AJ276292	AJ487855	AJ277989	U66088	U60282	AF235001
cDNA size (bp)	3,242	2,462	2,400	2,449	2,490	2,839	2,280
ORF (number of nucleotides)	1,932	1,914	1,998	1,980	1,932	1,857	1,857
Polypeptide chain (number of residues)	643	637	665	659	643	610	618

Comparison with published data on human (5), rat (4), and mouse (7) NIS.

A

	10	20	30	40	50	60	70	80
pNIS-D	MATVEGGARA	TEGAWDYGVF	ALMELVSTGI	GLWVGLARGG	QRSAEDFFTG	GRRLTAVFVG	LSLSASFMSA	VOVLGVPAEA
hNIS	-EA--T-E-P					A-L		S
rNIS	-EGA-A---				D--	Q-A	A	
mNIS	-EGA-A---				D--	Q-A	A	
	90	100	110	120	130	140	150	160
pNIS-D	YRYGLKFLWM	CLGQILNSLI	TALLFLPVFY	RLGLTSTYQY	LELRFSRAVR	LCGTLOYLVA	TMLYTGVIY	APALILNQVT
hNIS		V	M	E	M	I	I	
rNIS	A	A	F	I		I	I	
mNIS	A	V	L	I		I	I	
	170	180	190	200	210	220	230	240
pNIS-D	GLDIWAISLES	TGLICTEYTT	VGGMKAVIWT	DVFQVLVMT	GFWVLARGT	VLVGGPCRVL	ELAKNHSRIN	LMDFOLDPRR
hNIS		A	V	V	S	V	RQ	T-Q
rNIS		L	V	V	I	V	IL	RN
mNIS		L	V	V	V	V	M	WN
	250	260	270	280	290	300	310	320
pNIS-D	RYTEWTFVVG	GTLVWLSMYG	VNQAQVQRYV	ACRTEKQAKL	ALLINQVGLF	LIVSSAAACG	IYMFALYVDC	DPLLAGHISA
hNIS						C	VF	T
rNIS				H-GK	V-L	A-C	VY	K
mNIS		V-S		H-RK	V-L	A-C	VX	K
	330	340	350	360	370	380	390	400
pNIS-D	PDQYMLLV	DIFEDLPQVP	GLFLACAYSG	TLSTASTSIN	AMAAVTVDL	IKPRLPNLAP	RRLVIISKGL	SLIYGSACLT
hNIS						RS	K	
rNIS						M-G	K-F	F
mNIS						M-S	K-F	F
	410	420	430	440	450	460	470	480
pNIS-D	VAALSSLLGG	GVLQGSFTVM	GVTSGPLIGA	FVLGMELPSC	NTSGVLSGLA	AGLALSILWA	VGASLYPPSA	QSMGVLPSSA
hNIS			I	A	P	A-G	L-T	E
rNIS			T-L	A	P	V	T	GE
mNIS			T-L	A	P	V	T	GE
	490	500	510	520	530	540	550	560
pNIS-D	AGCALPTANA	SGLQDPVL*A	VNASSTASSL	ETDPEQPILA	ASFYAIISYLY	YGALGTLSTI	LCGALISCLT	GPTRKSALGP
hNIS	-R-VALSV--	-L--A-LP	A-D--R-P-S	GM-ASR-A-	D	T-V		T-A
rNIS	---T***-D	-V-LG-PG*	T---NGIP-S	GM-TGR-A-	DT	T-M		S
mNIS	---T***-A	-V-PS-PG*	A-T-RGIP-S	GM-SGR-AF	DT	T-M		S
	570	580	590	600	610	620	630	640
pNIS-D	GLLWDLTRQ	TASVAPKEEV	AALDDSLMKQ	GAEELPLAIK	KPPDFLSTNE	DHLLFLGQKE	VNGASSKTPG	SEHDKGHDLR
hNIS	-----A--	-----I--	N-V*-P	-----G-P--	-----R-F-----	LE--G-W--C	VG--G-R-QQ	
rNIS	-----A--	-----DT	T-EE-V*-P	-----DI-AVT	-----GLKPGA-	T-P-Y--H**	*****DV	
mNIS	-----A--	-----DT	TT-ED-V*-P	-----DI-AAT	-----GLKPGA-	T-P-Y--H**	*****DV	
pNIS-D	ETDL							
hNIS	--N-							
rNIS	--N-							
mNIS	--N-							

B

	620	630	640	650	660	670	680	690
pNIS-D	DHLLFLGQKE	VNGASSKTPG	SEHDKGHDLR	ETDL	643			
pNIS-F			*****	*****GC	637			
pNIS-J		*****	*****	*****DAEAWK	GALTWPRSQS	QPETHGEQLS	FLHPLPVFLP	VEGQTSHML 665

C

	390	400	410
pNIS-J	RRLVIISKGL	SLIYGSACLT	VAALSSLLGG
pNIS-ΔJ	*****	*****	*****

FIG. 2. Amino acid sequences deduced from the cloned porcine NIS cDNAs. A, Comparison between porcine (pNIS-D), human (hNIS), rat (rNIS), and mouse (mNIS) sequences. The upper numbers refer to the amino acid number of the porcine NIS, pNIS-D isoform. Identical amino acids are marked with a hyphen (-). Gaps created to maximize the alignment are indicated by asterisks. The 13 potential transmembrane domains are delineated in gray boxes. The putative cAMP-dependent protein kinase phosphorylation site located at position 554-557 conserved in human, rat, and mouse but not in pig is identified by empty boxes. The three conserved asparagine residues in N-glycosylation consensus sequences located at position 225, 489, and 501 are indicated by light-faced letters. B, Comparison of the C-terminal sequences of pNIS-D, pNIS-F, and pNIS-ΔJ composed of 643, 637, and 665 amino acids, respectively. C, Location of the six-amino-acid deletion found in pNIS-ΔJ.

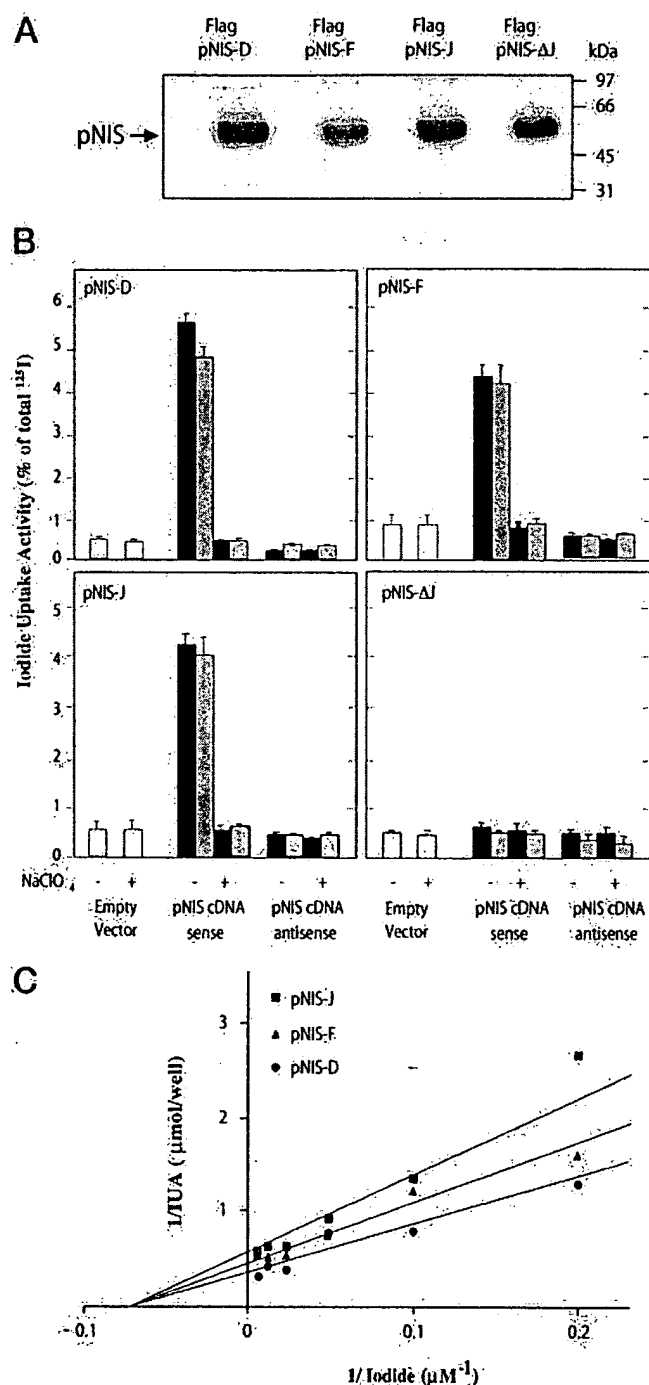


FIG. 3. Functional analyses of pNIS cDNAs by transient expression in Cos-7 cells. Cos-7 cells were transfected with the pTarget vector containing the pNIS-D, F, J, or ΔJ cDNA either wild-type or fused in 5' with the Flag sequence, in either the sense or the antisense orientation. Cos-7 cells, not transfected or transfected with the empty pTarget vector were used as controls. **A**, Expression of N-Flag pNIS isoforms by Cos-7 cells. Forty-eight hours after transfection, Cos-7 cells transfected with the tagged constructs were lysed and used to prepare membrane fractions that were analyzed by Western blot using the anti-Flag M2 monoclonal antibody. The amount of membrane protein loaded in each lane was 40 μg. A single band of about 62 kDa was detected in each case. The position of marker proteins of known molecular mass (expressed in kDa) is indicated on the right

Relationship between isolated pNIS cDNAs and thyroid pNIS transcripts

Complementary approaches including Northern blot, RT-PCR, and restriction analyses were used to establish a correspondence between isolated cDNA clones and porcine NIS transcripts expressed by pig thyrocytes in primary culture. As shown in Fig. 1, probe A (used for the isolation of the four cDNA clones) hybridized with transcripts of 3.5 and 3 kb. The relative abundance of the two bands did not vary with the culture conditions; they were 1) absent in cells cultured without TSH for 4 d, 2) present in high amounts when cells were cultured with TSH from the time of cell seeding, and 3) present at intermediate levels when cells were first cultured without TSH for 20 h and then activated by TSH for 3 d (Fig. 5A). Estimation of the ratio of these two bands in different experiments indicates that the amount of the 3.5-kb species was on average 1.7-fold higher than that of the 3-kb species. To demonstrate that pNIS-D clone actually corresponded to the 3.5-kb transcript (as suggested by their respective size), total RNA from cultured thyrocytes was subjected to Northern blot analysis using probe B, which specifically hybridized with pNIS-D cDNA. As expected, only the 3.5-kb transcript was detected. Consequently, the 3-kb transcript might possibly correspond to pNIS-F, J, and ΔJ clones lacking 492 bp, 538 bp, and 538 + 18 bp, respectively (as depicted at the top of Fig. 5). To identify the presence of mRNA corresponding to pNIS-F, J, and ΔJ, within the 3-kb molecular species, we designed two sets of primers (α1/α2 and β1/β2) flanking the deleted regions, as shown in Fig. 5B. The α1/α2 primers should allow one to distinguish the transcript corresponding to pNIS-F from those corresponding to pNIS-J and pNIS-ΔJ cDNAs. Accordingly, two fragments of 295 and 249 bp were generated by RT-PCR from pig thyrocyte RNA. As controls, amplicons of the same sizes were generated by PCR from pNIS-F and pNIS-J cDNA vectors, respectively (Fig. 5B). The identity of the products amplified by RT-PCR and PCR was ascertained by restriction analyses (data not shown). Thus, the 3-kb band probably contained mRNA species corresponding to clones pNIS-F and pNIS-J and/or pNIS-ΔJ. It was estimated (from several experiments) that pNIS-F mRNA was six to eight times more abundant than pNIS-J plus pNIS-ΔJ mRNAs. To discriminate between mRNAs deriving from pNIS-J and pNIS-ΔJ which differ by only 18 nucleotides, we used the primers β1/β2 for PCR amplification and a digestion at a BpuI site only present in pNIS-J. Products amplified by PCR from vectors containing pNIS-J and pNIS-ΔJ cDNA were slightly different in size (expected size: 393 and 377 bp) and the amplicon generated

side of the panel. **B**, Functional analysis of pNIS isoforms. Forty-eight hours after transfection, Cos-7 cells were analyzed for their capacity to concentrate iodide as indicated in *Materials and Methods*. The IUA was measured in the absence (–) or in the presence (+) of 0.1 mM NaClO₄ (an inhibitor of the NIS-mediated active transport of iodide). Incubations were made in triplicate. Results are expressed in percent of total $^{125}\text{I}^-$ iodide added to the culture medium. Columns and vertical bars represent the mean and SEM. Black columns, Wild-type cDNA; gray columns, cDNA fused to the Flag sequence. **C**, IUA of Cos-7 cells expressing pNIS-D, pNIS-F or pNIS-J as a function of medium iodide concentration (ranging from 5–80 μM). Data are presented as a double reciprocal plot. Each symbol represents the mean of triplicate.

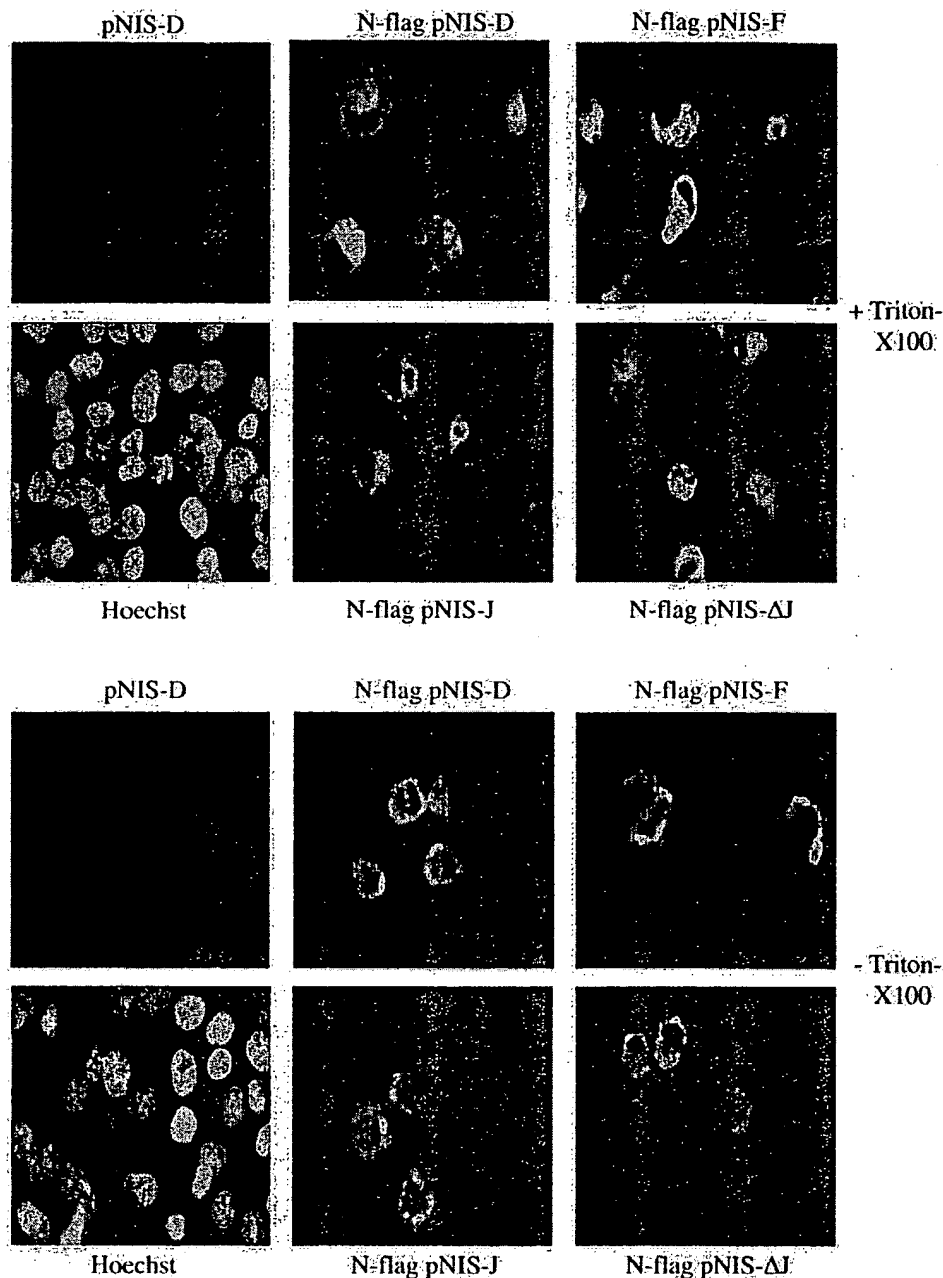


FIG. 4. Location of pNIS isoforms expressed by Cos-7 cells. Indirect immunofluorescence studies were based on the detection on the Flag epitope tag. Forty-eight hours after transfection, Cos-7 cells transfected with the pTarget vector containing the pNIS-D, F, J, or Δ J cDNA fused in 5' with the Flag sequence, were fixed and either not treated or treated with 1% Triton X-100 for plasma membrane permeabilization. Cells were then incubated with the anti-Flag M2 monoclonal antibody and a FITC-conjugated antimouse IgG secondary antibody. Cos-7 cells expressing the untagged pNIS-D were used as controls. Nuclear DNA was stained with the Hoechst reagent.

from pNIS-J was cleaved by BpuI treatment, whereas that from pNIS- Δ J remained intact. RT-PCR from total RNA from cultured thyrocytes yielded a band migrating as the 393-bp fragment, which was almost completely digested by BpuI. This result suggests that, if present, pNIS- Δ J mRNA was of very low abundance. It must be noticed that the amplification of pNIS- Δ J mRNA species could be understated, as the most abundant transcripts, pNIS-D and pNIS-F were also amplified using β 1/ β 2 primers. From the semiquantitative measurements of the relative abundance of the 3.5-kb and 3-kb transcripts on Northern blots and the relative abundance of pNIS-F, pNIS-J, and pNIS- Δ J-related 3-kb transcripts by RT-PCR, one can estimate that the transcripts corresponding to

clone D, F, J, and Δ J could account for about 60, 35, 5, and less than 1% of total pNIS mRNA.

Origin of the different pNIS isoforms

To identify the mechanism whereby several transcripts and consequently several NIS proteins differing in their C-terminus are generated, we analyzed the porcine genomic DNA corresponding to the 3' part of the NIS gene. Primers were designed on the basis of the known structure of the hNIS gene consisting of 15 exons interrupted by 14 introns (5). Porcine genomic DNA regions spanning exon 8 to exon 10 and exon 14 to exon 15 (numbering of exons refers to hNIS gene) were amplified, cloned, and sequenced. The exon/

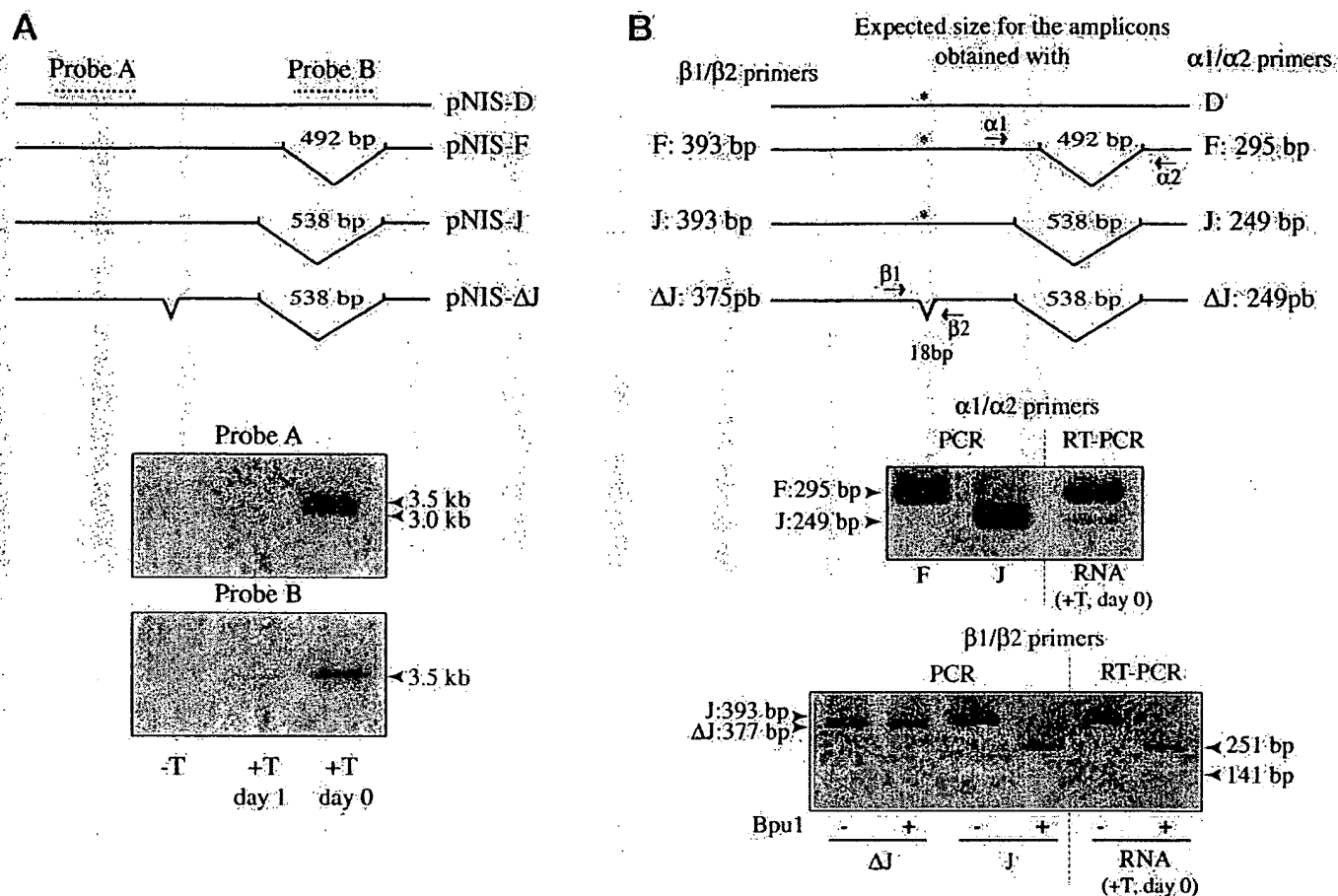


FIG. 5. Relationship between pNIS cDNA clones (D, F, J, and ΔJ) and pNIS mRNA variants expressed by porcine thyroid cells. A, Analysis by differential hybridization on Northern blot. Total RNA (25 μ g) extracted from porcine thyroid cells cultured for 4 d in the absence of TSH (-T) or in the presence of TSH added at d 1 of culture (+T, d 1) or added at the time of cell seeding (+T, d 0) was analyzed by Northern blot using either probe A or probe B. The dotted lines placed above cDNA sequence schemes give the position of each probe. Probe A should recognize all transcripts, whereas probe B should only hybridize with the transcript(s) corresponding to pNIS-D cDNA. The arrows identify pNIS transcripts by their size. B, RT-PCR and restriction analyses. Total RNA (1.5 μ g) from cultured cells (+T, d 0) and pNIS-F, J, or ΔJ cDNA (250 ng) were subjected to RT-PCR and PCR, respectively, using two sets of primers: $\alpha 1/\alpha 2$ and $\beta 1/\beta 2$ flanking the alternatively spliced regions as schematized at the top of the panel. The size of the amplicons that would result from the amplification of each transcript by RT-PCR or cloned cDNA by PCR is given on the left and the right sides of the schemes. PCR products (resulting from 30 amplification cycles) were separated on 2% agarose gel and visualized with ethidium bromide staining. The 393-bp PCR products generated from pNIS-D, pNIS-F, or pNIS-J contain a natural *Bpu*1 site that produces two fragments of 251 bp and 141 bp by restriction analysis; by contrast, the 377-bp amplicon generated from pNIS- ΔJ should remain undigested by *Bpu*1.

intron boundaries of the *pNIS* gene were established on the basis of consensus spliced signal sequences according to the gt/ag rule for RNA splicing (23), and the sequence homologies between the four pNIS cDNA clones schematically illustrated in Fig. 6. Two alternative splice donor sites (GT1 and GT2) and one alternative splice acceptor site (AG1) located within the last exon (exon 15 by reference to *hNIS* gene) were unequivocally identified. The use of GT2/AG1 splice sites allows the skipping of the stop codon operating in the primary transcript and generates pNIS-F that differs from pNIS-D by a 492-nucleotide deletion in the 3' translated and untranslated region (segment 2). The deletion does not alter the reading frame and the use of a downstream stop codon results in a protein shorter than pNIS-D by 6 residues (637 instead of 643 amino acids) (Fig. 6A, Table 1). The use of GT1/AG1 splice sites leads to a deletion of the same region

plus an additional upstream sequence of 46 nucleotides (segment 1) and results in a frame shift that produces pNIS-J with an extended (665 amino acids instead of 643 or 637 for forms D and F) and markedly different C terminus. A second alternative splice acceptor site (AG2) was found in exon 10. The use of the regular donor splice site of exon 9/intron 9 boundary and the acceptor site AG2 (Fig. 6B) leads to a deletion of 18 nucleotides within exon 10 (segment 3), which does not alter the reading frame and results in a six-amino-acid shorter pNIS-J protein (659 instead of 665 amino acids). All the alternative splicing events identified in the porcine species would specifically occur in that species. Indeed, the donor splice sites GT1 and GT2 and the acceptor splice sites AG1 and AG2 are either absent or present in inappropriate combination (to generate splice variants) in human, rat, or mouse *NIS* genes (Fig. 7).

last intron

gt ag

GT1 GT2 AG1

pVIS gene: =

pVIS-D: =

pVIS-J: =

pVIS-F: =

TAA

GT1 AG1

segment 1 Δ538

GT2 AG1 TGA

segment 2 Δ492

Last intron Last exon **[75]**
 Gene ctttctctctcttccccctagcagggctctgaggaattgcctttggcaatcaagagccctcttgacttccctgtccactaatgaggaccatctgctctttctggggcagaaggaggtgaatggagccagctccaagacccaggcagt
 pNIS-Dggtgctcaggaattgcctttggcaatcaagagccctcttgacttccctgtccactaatgaggaccatctgctctttctggggcagaaggaggtgaatggagccagctccaagacccaggcagt
 pNIS-Jggtgctcaggaattgcctttggcaatcaagagccctcttgacttccctgtccactaatgaggaccatctgctctttctggggcagaaggaggtgaatggagccagctccaagacccaggcagt
 pNIS-Fggtgctcaggaattgcctttggcaatcaagagccctcttgacttccctgtccactaatgaggaccatctgctctttctggggcagaaggaggtgaatggagccagctccaagacccaggcagt
 Segment 1
[72]
 Gene GAACATGACAAAGGTCATGACCTGCCTGAGAGGACCTCTAAGCGGGATGGACCACTCGGATGGAACCTCAGGGGGGCCAATCCAGCGCCCAAGGCCACAGGGTTAGCGGTTAGGGTTAGGGTTAGGGTTCAGAGG
 pNIS-D GAACATGACAAAGGTCATGACCTGCCTGAGAGGACCTCTAAGCGGGATGGACCACTCGGATGGAACCTCAGGGGGGCCAATCCAGCGCCCAAGGCCACAGGGTTAGCGGTTAGGGTTAGGGTTCAGAGG
 pNIS-J
 pNIS-F Segment 2
 gene GGCCAAATCCCAAGCCCCCTGGCTGGACCAATCATATGCAAAATGGGTTTGAGACTGTGTGTCCCACTTACCGGAAGAGGTGAAGCCCCACCTCCAGAAGGTCATATAATCAGCCCTCCTGCTTCTAGCCCCTAATCTTAGATCTG
 pNIS-D ggccaatcccaaggccccctggctggaccacatcatatgcaaatgggtttgagactgtgtgtcccaaccttacoggaagaggtgaagcccaacctccagaaggtcatataatcgacctcctgcttctagccccatcttagatgctg
 pNIS-J
 pNIS-F
 gene CACCCTGCCCTCTCTCTAAACAAAGCCAGACTTTTCTCTAACTACAGTATGAAGTGTGTAGATCTCTCTGGACAACATGGGAAACCCAGGCTCCACCTTGGGCTCTGAGAAATATCCAGCTCTTCCCTGGGGTTGGGGG
 pNIS-D caccctgccccctctctctaaacaaaggccagactttctctaaactacagtatgaagttgttagatcctctctggacaacatggggaaccccaaggctccacctggggtctgagaaatatccagctcttccctggggggttggggg
 pNIS-J
 pNIS-F
[62]
 gene GGGATCTGAATCTTAGCAATGATTTTGAGGCTCTGAGGCCAACCCCTATACCCACTTTACAGTGGATGCTGAGGCTCTGGAAGGGGCATTGACTTGGCCAAGGTCACAAAGCCAACAGAGACTCATGGAGAGCAGCTCTCTCTC
 pNIS-D gggatctgaatcttagcaatgattttgagggtcctgaggeccaaacccctatacccaactttacaggtggaatgctgagggcctggaaaggggcattgacttggccaaggtcacaaagccaacagagactcatggagagcagctctctctc
 pNIS-JGTGGATGCTGAGGCTCTGGAAGGGGCATTGACTTGGCCAAGGTCACAAAGCCAACAGAGACTCATGGAGAGCAGCTCTCTCTC
 pNIS-FGTGGATGCTGAGGCTCTGGAAGGGGCATTGACTTGGCCAAGGTCACAAAGCCAACAGAGACTCATGGAGAGCAGCTCTCTCTC

[illegible]

Fig. 6. Identification of alternative donor and acceptor sites on porcine *NIS* gene. Schematic representation and alignment of nucleotide sequences of the porcine *NIS* genomic DNA and *NIS* cDNA segments corresponding to 1) the last intron and last exon (putative intron 14 and exon 15 by reference to the human *NIS* gene organization) (A) and 2) exon 9 to exon 10 interrupted by intron 9 (B). Exons are represented as gray boxes, and introns as thin lines. The arrowheads indicate the stop codons, and empty boxes represent the 3' untranslated regions of each splice pNIS cDNA variants (D, F, and J). Intronic and 3'-untranslated region sequences are written in lowercase letters; exon (gene) and translated (cDNA) sequences are written in capital letters. Donor (GT1, GT2) and acceptor (AG1, AG2) sites are in bold type either as lowercase (intron sites) or capital letters (alternative splice sites in exons). Dotted boxes and dotted lines indicate the deleted segment in each splice pNIS cDNA variants. The GenBank accession numbers to the intronic nucleotide sequence data reported in this paper are AJ306406, AJ306407, and AJ487979.

this transcript by RT-PCR amplification from thyroid cell RNA is in keeping with this hypothesis. Finally, information drawn from computer analyses appears in full agreement with the experimental data.

The alternative splicing events occurring on pNIS transcripts are probably not subjected to regulation. Indeed, the relative proportion of the 3.5- and 3-kb molecular species as well as the relative proportion of the pNIS-F and J transcripts (within the 3-kb band) did not markedly vary in thyroid cells upon generation of transcripts following TSH activation of transcription or upon disappearance of transcripts following TSH withdrawal. Despite the presence of TSH, the pNIS transcript level markedly decreased within the first 24 h of culture of freshly dispersed porcine thyrocytes. This decline does not seem to be related to a period of TSH refractoriness that could result from TSH receptor alterations caused by the proteolytic enzyme treatment used for cell isolation. Indeed, within the same period of time, thyrocytes readily respond to TSH in forming cell aggregates from which thyrocytes reconstitute follicle structures. The decline in NIS transcript level that was similar in the presence and in the absence of TSH, would rather reflect a transient and probably selective decrease of NIS gene transcription or increase of mRNA degradation; we observed that the level of transcripts of other thyroid-specific genes, thyroglobulin, Pax-8 did not significantly change during the same period of time.

Recombinant proteins expressed in Cos-7 cells from pNIS-D, F, J, and ΔJ clones had a molecular mass (~62 kDa) lower than that expected from available data on rNIS, mNIS, and hNIS. Indeed, in these species, both thyroid NIS and recombinant NIS proteins (expressed in Cos-7 or CHO cells) are glycoproteins with an apparent molecular mass ranging from 80–90 kDa (7, 11, 15, 24). Using anti-pNIS antibodies recently generated in our laboratory, we found that thyroid pNIS had the same molecular mass as murine NIS or hNIS and that its deglycosylation using *N*-glycosidase F led to 60- to 65-kDa polypeptides (Trouttet-Masson, S., F. Bernier-Valentin, S. Selmi-Ruby, and B. Rousset, unpublished data). Thus, the recombinant pNIS proteins produced from Cos-7 cells in this study correspond to nonglycosylated proteins. This is very likely due to a limitation of Cos-7 cells that we used; indeed, we observed a similar lack of glycosylation for another recombinant protein expressed by the same cells. Although performed on nonglycosylated proteins, our functional data should be fully reliable because Carrasco and her colleagues (11) have convincingly demonstrated that glycosylated, partially glycosylated, and nonglycosylated rNIS had the same activity and iodide transport kinetics.

Recombinant proteins expressed from pNIS-D, F, and J (for which the natural mRNAs have been firmly identified) differ in their C terminus and are all endowed with a function of active iodide transporter eliciting very close, if not identical, kinetics. These data indicate that the sequence and/or the length of the C-terminal intracellular domain of NIS (at least the last 23 amino acids) are not determinant for the activity of the protein. A similar conclusion could be drawn from the sequence comparison between hNIS and murine NIS. Indeed, compared with hNIS, the functional rNIS and mNIS lack 18 of the last 24 amino acids. The immunofluorescence detection of the flag epitope positioned at the N-

terminal end of the recombinant pNIS proteins, without membrane permeabilization, confirms that the NIS protein has its N terminus outside the cells.

The characterization of a nonfunctional NIS protein (pNIS-ΔJ), deleted of 6 amino acids (391–396) in the putative transmembrane domain 10, suggests that at least one of these amino acids is important for the ability of the protein to transport iodide. This observation is in keeping with data obtained in patients with congenital hypothyroidism in which a missense mutation G395R on hNIS causes an iodide transport defect (25). Like the pNIS-ΔJ protein, the G395R hNIS mutant protein was properly targeted to the plasma membrane of Cos-7 cells. In terms of structure-activity relationship, it is worth noticing that the residues with a potential importance for the function and/or the stability of the protein (26, 27) corresponding to identified mutations of hNIS in position: G93, Q267, C272, T354, that cause a thyroid iodide transport defect and congenital hypothyroidism, are conserved in the three functional pNIS proteins. The nonfunctional pNIS-ΔJ protein did not exert any dominant negative effect in cotransfection experiments with one or the other active isoforms.

At the moment, we do not know whether the existence of several active NIS protein isoforms in the porcine species instead of only one in the human, rat, and mouse species could have physiological implications for the thyroid iodide economy. We already know that protein isoforms do not differ in their affinity for iodide. Production of antibodies capable of discriminating the three pNIS protein isoforms would allow one to analyze whether they have distinct cellular properties.

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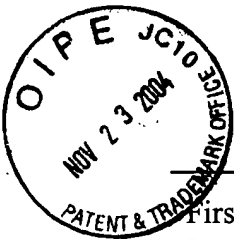
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RECOMBINANT PORCINE LEPTIN REDUCES FEED INTAKE AND STIMULATES GROWTH HORMONE SECRETION IN SWINE

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Two experiments (EXP) were conducted to test the hypothesis that porcine leptin affects GH, insulin-like growth factor-I (IGF-I), insulin, thyroxine (T_4) secretion, and feed intake. In EXP I, prepuberal gilts received intracerebroventricular (ICV) leptin injections. Blood was collected every 15 min for 4 hr before and 3 hr after ICV injections of 0.9% saline (S; $n = 3$), 10 μg ($n = 4$), 50 μg ($n = 4$), or 100 μg ($n = 4$) of leptin in S. Pigs were fed each day at 0800 and 1700 hr over a 2-wk period before the EXP. On the day of the EXP, pigs were fed at 0800 hr and blood sampling started at 0900 h. After the last sample was collected, feeders were placed in all pens. Feed intake was monitored at 4, 20, and 44 hr after feed presentation. In EXP II, pituitary cells from prepuberal gilts were studied in primary culture to determine if leptin affects GH secretion at the level of the pituitary. On Day 4 of culture, 10^5 cells/well were challenged with 10^{-12} , 10^{-10} , 10^{-8} , or 10^{-6} M [Ala¹⁵]-h growth hormone-releasing factor-(1-29)NH₂ (GRF), 10^{-14} , 10^{-13} , 10^{-12} , 10^{-11} , 10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} , or 10^{-6} M leptin individually or in combinations with 10^{-8} and 10^{-6} M GRF. Secreted GH was measured at 4 hr after treatment. In EXP I, before injection, serum GH concentrations were similar. Serum GH concentrations increased ($P < 0.01$) after injection of 10 μg (21 ± 1 ng/ml), 50 μg (9 ± 1 ng/ml), and 100 μg (13 ± 1 ng/ml) of leptin compared with S (1 ± 2 ng/ml) treated pigs. The GH response to leptin was greater ($P < 0.001$) in 10 μg than 50 or 100 μg leptin-treated pigs. By 20 hr the 10, 50, and 100 μg doses of leptin reduced feed intake by 53% ($P < 0.08$), 76%, and 90% ($P < 0.05$), respectively, compared with S pigs. Serum IGF-I, insulin, T_4 , glucose, and free fatty acids were unaffected by leptin treatment. In EXP II, relative to control (31 ± 2 ng/well), 10^{-10} , 10^{-8} , and 10^{-6} M GRF increased ($P < 0.01$) GH secretion by 131%, 156%, and 170%, respectively. Only 10^{-6} M and 10^{-7} M leptin increased ($P < 0.01$) GH secretion. Addition of 10^{-11} and 10^{-9} M leptin in combination with 10^{-6} M GRF or 10^{-11} M leptin in combination with 10^{-8} M GRF-suppressed ($P < 0.05$) GH secretion. These results indicate that leptin modulates GH secretion and, as shown in other species, leptin suppressed feed intake in the pig. © Elsevier Science Inc. 1998

INTRODUCTION

The recently discovered protein, leptin, suppresses feed intake and stimulates metabolic rate and reproductive function in ob/ob genetically obese mice (1,2). Leptin receptors are expressed in the ventromedial and arcuate regions of the hypothalamus, pituitary, adipose tissue, ovary, and some additional organs (3,4). The two primary regulators of growth hormone (GH) secretion, GH-releasing factor (GRF), and somatostatin (SS), are produced in the arcuate and ventromedial hypothalamus in the pig brain (5). These same areas are also involved in food intake regulation. Thus, leptin may regulate feed intake and growth

in swine. Perhaps alteration in leptin secretion and/or receptors in growing swine could be used to enhance feed intake and growth. Porcine leptin was recently cloned and synthesized (Ramsay and coworkers, unpublished observations). Therefore, two experiments (EXP) were conducted to test the hypothesis that leptin modulates feed intake, GH, insulin-like growth factor-I (IGF-I), insulin, and thyroxine (T_4) secretion in the pig.

MATERIALS AND METHODS

Experiment (EXP) I. Eight crossbred prepuberal gilts, 80.6 ± 2.7 kg body weight (BW) and 150 d of age were implanted surgically with lateral intracerebroventricular (ICV) cannulas using the stereotaxic procedure of Estienne et al. (6) and Barb et al. (7). Animals were individually penned in an environmentally controlled building and exposed to a constant temperature of 22° C and artificial 12:12-hr light:dark photoperiod. Pigs were meal-fed daily at 0800 and 1700 hr a corn-soybean meal ration (14% crude protein) supplemented with vitamins and minerals, according to the National Research Council guidelines (8). One week after the last ICV surgery, all pigs were fitted with an indwelling jugular vein cannulae (9) 24 hr before treatment. On the day of the EXP, pigs were fed at 0800 hr and blood sampling started at 0900 hr. Blood samples were collected every 15 min for 4 hr before and 3 hr after ICV injections of 150 μ l 0.9% saline (S), or 10, 50, or 100 μ g of recombinant porcine leptin in 150 μ l of S. One week later, the EXP was repeated with pigs reassigned to treatment such that no pig received the same dose as before, resulting in four pigs/dose of leptin or S. One S animal was eliminated from the study because of loss of patency of the jugular cannula. Serum was harvested and stored at -20° C until assayed for GH by radioimmunoassay (RIA). Hourly samples were assayed for IGF-I, T_4 , insulin, glucose, and free fatty acids (FFA).

Feed Intake. After the last sample was collected, feeders were placed in all pens and feed intake monitored at 4, 20, and 44 hr after feed presentation.

Expression and purification of the recombinant porcine leptin. Porcine leptin cDNA sequence representing the secreted porcine protein (amino acids 22 to 167) was amplified by PCR from a porcine leptin cDNA containing plasmid (PCR2.1) (Invitrogen, Carlsbad, CA) harboring the complete coding region (Ramsay et al., Genbank Accession No. U59894). The cDNA was subcloned in-frame into a procaryotic expression vector pGEX-2T (Pharmacia Biotech, Piscataway, NJ) and confirmed by DNA sequencing. The DNA construct was then transformed and overexpressed in *Escherichia coli* (JM109) after induction by IPTG. A crude protein extract containing the GST-leptin fusion protein was prepared by conducting sonification and precipitation. Sarkosyl and Triton X-100 were added to aid solubilization of the protein. Filtered protein extract was loaded onto a prepacked Glutathione Sepharose 4B column and washed with 30-bed volumes of PBS. The column was then incubated with thrombin solution (20 NIH units per liter culture in cleavage buffer, 20 mM Tris-HCl, pH 7.9, 140 mM NaCl, 10% Glycerol, 2.5 mM $CaCl_2$) according to the manufacturer's recommendations. Recombinant leptin protein was finally recovered in the flow through and the subsequent washes. Refolding of the protein was achieved by denaturing the protein in 4 M urea and dialyzing it successively against 3, 2, 1, and 0.5 M urea and three changes of PBS. Each dialysis step was performed for 12 hr in 10,000 MW cutoff tubing at 4° C against 50 volumes of solution. Purity of the recombinant protein was estimated by staining a SDS-PAGE gel with Coomassie Blue. Recombinant leptin was the only band detectable on the gel.

EXP II. Pituitary cells were studied in primary culture to determine if leptin affects GH secretion at the level of the pituitary. Two studies were conducted with six pituitary glands/study from crossbred prepuberal gilts 180 d of age and weighing approximately 105 kg. Pituitary glands were removed aseptically and ovaries were examined at slaughter.

Gilts were considered prepuberal because their ovaries were devoid of corpora albicantia and corpora lutea. All subsequent procedures were performed under sterile conditions. The anterior lobe was dissected from each pituitary gland, cells were enzymatically dispersed and cultured as described previously (10). Briefly, after cells were dispersed and centrifuged, the cell pellet was resuspended in growth medium (Dulbecco's Modified Eagle's medium [DME] Ham's Nutrient mixture F-12 [F-12]) 90% [vol/vol; 4,150 mg/l glucose], and 10% [vol/vol] Fitton-Jackson medium [BGjb] (Gibco, Grand Island, NY) containing 2 mg/ml of BSA, 10 mM HEPES [pH 7.2], 100 U/ml of penicillin, 250 ng/ml of amphotericin B (Sigma Chemical Company, St. Louis, MO), and 100 μ g/ml of streptomycin to which was added 2% [vol/vol] fetal bovine serum (Pharmacia) supplemented with 100 ng/ml of cortisol, 1.0 ng/ml of human insulin, 10 μ g/ml of human transferrin (Sigma), 10 pg/ml of glucagon, 100 pg/ml of epidermal growth factor, 200 pg/ml of bovine parathyroid hormone (PTH), 400 pg/ml of triiodothyronine (Sigma), and 200 pg/ml of fibroblast growth factor (Collaborative Research, Bedford, MA). Cell viability and number were assessed by counting cells which excluded trypan blue on a hemocytometer. Culture medium was changed on Day 3 (day of seeding = Day 0 of culture) and replaced with serum-free growth medium as described before, except using DME containing 1,000 mg of glucose/l (Sigma). On Day 4 of culture, medium was discarded, plates were rinsed twice with serum-free medium, and 10^5 pituitary cells/well were cultured in 1 ml fresh medium without serum. Cells were challenged with 10^{-12} , 10^{-10} , 10^{-8} , or 10^{-6} M [Ala¹⁵]-h GRF-(1-29)NH₂, 10^{-14} , 10^{-13} , 10^{-12} , 10^{-11} , 10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} , or 10^{-6} M leptin individually or in combinations with 10^{-8} and 10^{-6} M GRF. Cells were exposed to treatment for 4 hr, at which time the medium was harvested and quantified for GH. There were six wells per treatment per study.

Metabolite and Hormone Assays. Serum and media concentrations of GH were quantified by RIA (11). Assay sensitivity was 0.4 ng/ml. Intra- and inter-assay coefficients of variation were 3.2% and 13.6%, respectively. Serum IGF-I concentrations were quantified by RIA (12). Assay sensitivity was 0.01 ng/ml. Intra- and interassay coefficients of variation were 3.2% and 3.5%, respectively. Total serum T₄ and insulin concentrations were quantified by RIA Kits (INCSTAR Corporation, Stillwater, MN). Samples were assayed for glucose using a glucose oxidase kit (Sigma) and FFA using a colorimetric assay kit (Wako Chemicals USA, Inc., Richmond, VA).

Statistical Analysis. To determine the effect of leptin on serum GH, IGF-I, T₄, glucose, FFA, and insulin concentrations across time and feed intake in EXP I, data were subjected to the general linear model split plot-in-time ANOVA procedure of the Statistical Analysis System (13). The statistical model included dose, pig, time, and replicate. Effects of dose and replicate were tested using replicate \times dose as the error term. Replicate \times dose was tested using pig within dose \times replicate as the error term. Time and time \times dose were tested using dose \times time \times replicate as the error term. Differences between treatment means within a time were determined by least-squares contrasts (13).

In EXP II, data were converted to percentage of basal secretion before averaging to minimize differences between replicates. To obtain an estimate of variation between control wells, medium GH concentrations for control wells were converted to a percentage of mean basal GH concentration. This was then used to calculate a SE for basal secretion. Basal secretion (control; C) was the amount of hormone secreted into the culture medium per 10^5 cells seeded/well in the absence of a secretagogue. Converted data were tested for homogeneity of variance using Hartley's F max test (14). Data were then subjected to a one way ANOVA and differences between means were determined by least-squares contrast (13).

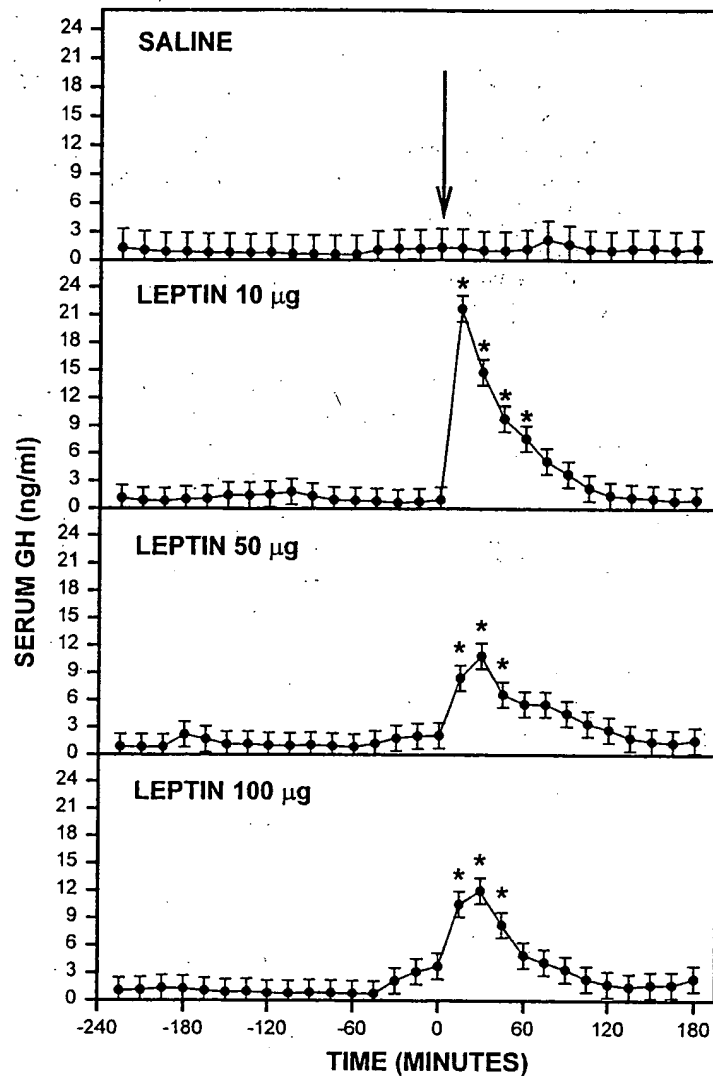


Figure 1. Serum GH concentrations (mean \pm SE) for pigs receiving ICV injection of saline ($n = 3$), 10 μ g ($n = 4$), 50 μ g ($n = 4$), or 100 μ g ($n = 4$) of leptin at time 0. Times at which effects of treatment were different from saline-treated animals are indicated * ($P < 0.01$).

RESULTS

EXP I. Before injection, serum GH concentrations were similar ($P > 0.1$) among groups and averaged 1.6 ± 1.5 ng/ml. Serum GH concentrations increased ($P < 0.01$) by 15 min after injection of 10 μ g (21 ± 1 ng/ml), 50 μ g (9 ± 1 ng/ml), and 100 μ g (13 ± 1 ng/ml) of leptin, compared with S (1 ± 2 ng/ml) treated pigs (Figure 1). The GH response to leptin was greater ($P < 0.001$) by 15 min after 10 μ g than after 50 or 100 μ g leptin. At 4 hr, feed intake was similar among groups. By 20 hr the 10, 50, and 100 μ g doses of leptin reduced feed intake by 53% ($P < 0.08$), 76%, and 90% ($P < 0.05$), respectively, compared with S pigs. By 44 hr, feed intake remained suppressed ($P < 0.05$) and averaged 62%, 66%, and 50% of control animals for the 10, 50, and 100 μ g doses of leptin, respectively (Figure 2). Serum IGF-I and T_4 were unaffected by leptin treatment

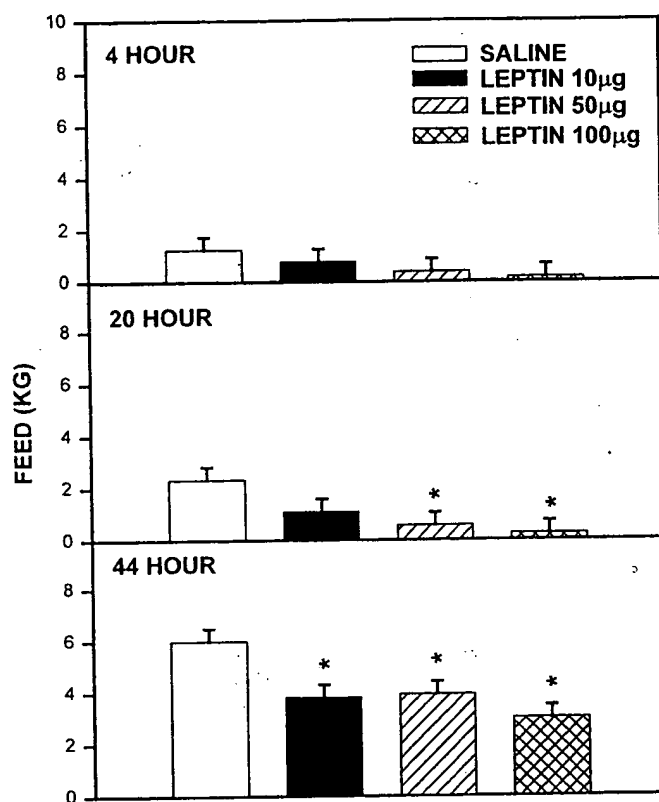


Figure 2. Cumulative feed intake (mean \pm SE) for pigs receiving ICV injection of saline ($n = 3$), 10 μ g ($n = 4$), 50 μ g ($n = 4$), or 100 μ g ($n = 4$) of leptin. Feed intake was monitored at 4, 20, and 44 hr after feed presentation. Times at which effects of treatment were different from saline-treated animals are indicated * ($P < 0.05$).

and averaged 63 ± 4 ng/ml and 43 ± 3 ng/ml, respectively, among the groups. Serum insulin concentration were similar among groups and averaged 26 ± 3 μ U/ml before treatment and 29 ± 4 μ U/ml by 1 hr after treatment. Serum glucose concentrations were similar among groups and averaged 89 ± 11 mg/dl. Serum FFA levels were unaffected by treatment and ranged from 141 ± 25 to 323 ± 29 μ Eq/l throughout the blood sampling period.

EXP II. Basal GH secretion (control; $n = 12$ wells) was 31 ± 2 ng/well. Relative to control at 4 hr, 10^{-10} , 10^{-8} , and 10^{-6} M GRF increased ($P < 0.01$) GH secretion by 131%, 156%, and 170%, respectively. Only 10^{-6} M (143%) and 10^{-7} M (147%) leptin increased ($P < 0.01$) GH secretion (Figure 3). Addition of 10^{-11} and 10^{-9} M leptin in combination with 10^{-6} M GRF or 10^{-11} M in combination with 10^{-8} M GRF suppressed ($P < 0.05$) GH secretion (Figure 4).

DISCUSSION

The discovery of the ob gene and anti-obesity effects of leptin seems to be a breakthrough in understanding the role adipose tissue plays in regulating food intake, body weight, and endocrine function. The intrinsic mechanisms through which leptin exerts its effects are poorly understood. It is currently hypothesized that leptin acts at the brain to reduce food intake, increase energy expenditure and alter endocrine activity (15,16). Leptin receptors are present in different hypothalamic nuclei that are involved in regu-

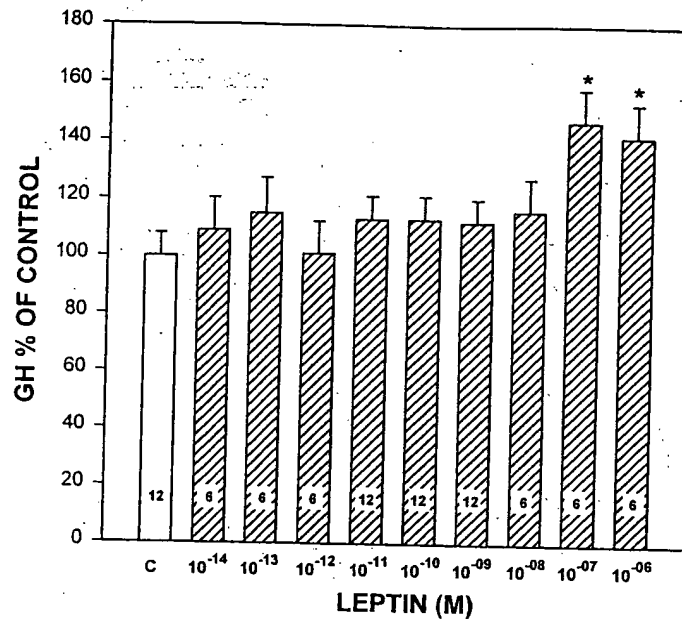


Figure 3. Effects of leptin on basal GH secretion. Values are the means \pm SE for the two studies. Numbers in columns = number of wells per treatment. Control (C) = basal secretion in the absence of treatment. *Different from C ($P < 0.01$).

lating pituitary hormone secretion (3), and several reports have demonstrated the effects of leptin administration on the hypothalamic pituitary axis. Leptin treatment increased plasma concentrations of LH, FSH, and testosterone in fasted mice as well as ob/ob mice (1,16,17). Lastly, a direct effect of leptin on LH, FSH, and prolactin release from rat

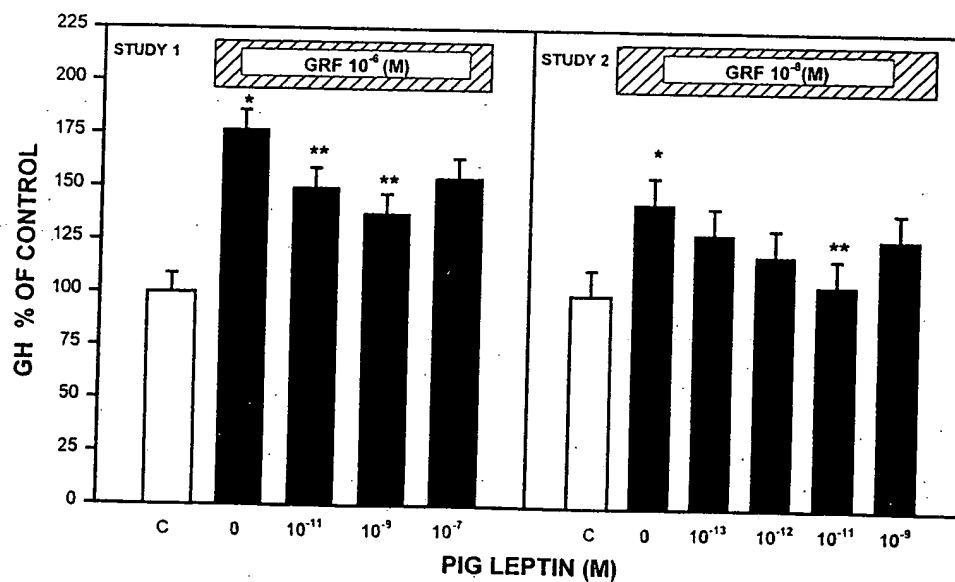


Figure 4. Interaction of leptin with GRF on GH secretion. Values are the means \pm SE for each study ($n = 6$ wells/treatment). Control (C) = basal secretion in the absence of treatment. *Different from C ($P < 0.01$). **Different from GRF alone ($P < 0.05$).

pituitaries in culture was reported (17). Collectively, these data indicate that leptin plays a role in regulating the hypothalamic pituitary axis as well as food intake.

The thyroid axis is also influenced by leptin. Twice daily administration of leptin for 48 hr increased serum T_4 levels in fasted mice compared with fed controls (16). In addition, ICV leptin administration for 4 d in normal fed rats decreased serum T_4 levels and increased serum triiodothyronine concentrations (Azain et al., 1997 unpublished observations). The failure of leptin treatment to alter T_4 secretion in the current study may, in part, be related to species differences, nutritional status, and/or acute versus chronic treatment. Perhaps serum leptin levels must exceed or fall below a critical threshold before change in the neuroendocrine axis can be initiated.

In the present study ICV administration of leptin resulted in a marked increase in GH secretion in normal fed pigs with maximum concentrations occurring at 15–30 min post-ICV injection, similar to GH response to exogenously administered GRF (18). This is in contrast to a recent report by Carro et al. (19) in which central administration of leptin failed to increase GH secretion in normal fed adult male rats, but did reverse inhibition of GH secretion exerted by fasting. This paradox between the studies may in part be related to species, age, and/or sex differences. For example, in the rat fasting suppressed GH levels (20), whereas in the pig fasting elevated serum GH concentrations (21). Moreover, age and sex have a profound influence on the pattern of GH secretion in the rat and pig (22–24). We suggest that stimulation of GH secretion by leptin is attributable to a reduction in hypothalamic NPY release and a concomitant increase in GRF and/or decrease in SS release. In support of this idea, hypothalamic NPY release is stimulated by food deprivation in rats (25). Leptin receptors are present on NPY neurons in the hypothalamus (26) and immunocytochemical evidence demonstrated synaptic connection between NPY neuronal projections and SS neurons (25). Central administration of NPY inhibited GH secretion and stimulated SS release from hypothalamic tissue in vitro (27).

This is the first report demonstrating a direct effect of leptin on pituitary GH secretion. Leptin receptors were demonstrated in the pituitary of sheep (4), suggesting a physiological role for leptin in regulating GH secretion. However, only the 10^{-7} and 10^{-6} M leptin increased GH secretion in vitro in this study. These doses could be considered supra physiological and may not be physiologically relevant. More importantly leptin at doses considered to be physiological, suppressed GRF induced GH secretion. Yu et al. (17) reported that leptin increased FSH and LH secretion from the rat anterior pituitary in vitro. Taken together these data suggest the presence of leptin receptors on the secretory cells of the anterior pituitary. Further work is needed to elucidate the physiological role of leptin in modulating pituitary function.

The demonstration that recombinant porcine leptin reduced feed intake in a dose-dependent manner provides further evidence for the hypothesis that adipose tissue secretes a protein signal that acts on the central nervous system to regulate feed intake (2,28). The action of leptin on feed intake was still apparent at 44 hr after feed presentation. Similar studies in rodents showed that leptin injection into the lateral or third ventricle reduced food intake for up to 24 hr (29,30). These data strongly imply that leptin acts directly within the central nervous system.

The GH response to leptin, in the present study, was greater for the 10 μ g dose whereas, for feed intake the response was greater for the 100 μ g dose. This dichotomy, in part, may be attributable to separate neural pathway(s) involved in GH secretion and food intake regulation. Leptin acts to reduce the activity of arcuate NPY neurons and decrease release of NPY in the paraventricular nucleus and other brain locations involved in feed intake regulation (31). Growth hormone-releasing factor, SS (5), and NPY are produced primarily in the arcuate nucleus and ventromedial hypothalamus in the pig (32). More-

over, leptin and NPY may also affect other types of neurons (33), such as those modulating the sympathetic nervous system. Therefore, it is conceivable that leptin acts by different neural pathways to inhibit food intake and stimulate GH secretion.

It is well established that the anabolic activity of GH is mediated by serum IGF-I and IGF-I concentrations are GH dependent (34). It was hypothesized that serum IGF-I concentrations would increase after the leptin-induced increase in GH secretion. The lack of an increase in serum IGF-I concentrations after leptin treatment may be related to the length of the blood sampling period. Sillence and Etherton (35) reported a 4–6 hr lag before serum IGF-I concentrations began to rise after exogenous GH administration. Similar findings have been reported in humans (36). Thus, since blood samples were only collected for 3 hr after leptin treatment, the GH induced increase in serum IGF-I levels would have occurred after the blood sampling period.

Understanding the mechanisms that regulate food intake is of great relevance in the rapidly growing pig. Swine do not eat to gut fill, but stop eating based on other signals and behavioral mechanisms (37). Growth studies demonstrated that these animals have not reached their genetic potential for lean deposition. Therefore, understanding the leptin/GH axis is imperative to develop new methods to promote maximal growth and muscle accretion.

We have shown here that central administration of leptin inhibited feed intake and stimulated GH secretion and altered pituitary response to GRF. This is consistent with the idea that leptin is an important link between metabolic status, neuroendocrine system, and the growth process. How leptin achieves this link is poorly understood. A more detailed examination of the physiological role of leptin in regulating growth in the pig will be dependent on monitoring metabolic hormones that are influenced by leptin and by measuring circulating concentrations of leptin and associated changes in the GRF/GH axis.

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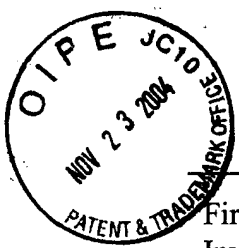
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First Named

Inventor : Michael E. Spurlock

Appln. No. : 09/932,888

Filed : August 20, 2001

Title : Porcine Leptin Protein, Antisense and Antibody

Docket No. : LL31.12-0016

Group Art Unit: 1647

Examiner: C. J. Saoud

EXHIBIT O

of

DECLARATION

submitted under 37 C.F.R. 1.132

E. Carro, R. Senaris, R.V. Considine, F.F. Casanueva, C. Dieguez, Regulation of In Vivo Growth Hormone Secretion by Leptin, Endocrinology, Vol. 138, No. 5, pages 2203-2206 (1997)

REGULATION OF IN VIVO GROWTH HORMONE SECRETION BY LEPTIN.

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ABSTRACT.

Leptin, the product of the *ob* gene, is a recently discovered hormone secreted by adipocytes that regulates food intake and energy expenditure. Growth hormone (GH) secretion is markedly influenced by body weight being markedly suppressed in obesity and underweight. The aim of the present study was to study whether leptin can act as a metabolic signal connecting the adipose tissue with the growth hormone axis. We administered leptin antiserum (10 μ l, i.c.v.) or normal rabbit serum (NRS; 10 μ l, i.c.v.) to freely moving fed rats. Furthermore we assessed the effect of leptin administration (10 μ g, i.c.v.) on fed and fasted rats. Spontaneous GH secretion was assessed over 6 hours with blood samples taken every 15 min. Administration of leptin antiserum led to a decrease in spontaneous GH secretion as assessed by the area under the curve (AUC) (168 ± 72 ng/ml/6h) in comparison to NRS-treated rats (813 ± 179 ng/ml/6h, $p < 0.01$). While leptin administration (10 μ g/rat; i.c.v.) to normal fed rats did not modify spontaneous GH secretion, leptin administration to fasted rats led to a reversal of the inhibitory effect exerted by fasting on GH secretion (AUC, 1650 ± 351 ng/ml/6h vs 77 ± 32 ng/ml/6h, $p < 0.01$). This data suggests that leptin is a metabolic signal that regulates GH secretion.

In addition to stimulating body growth, GH plays an important role in metabolism. In turn, alterations in nutritional status, such as obesity or food deprivation, markedly influence GH secretion. In obesity, GH secretion in either human subjects or in genetically obese rats has been found to be clearly impaired. Because GH secretion is normalized after weight loss in obesity or after refeeding in states of food deprivation, there is no doubt that altered GH secretion develops as a consequence of altered metabolic status (1). However the mechanisms by which metabolic status regulates GH secretion is not yet understood.

The *ob* gene is an adipocyte specific gene that encodes leptin, a protein that regulates body weight and energy expenditure (2-6). Recent data has clearly shown that the amount of leptin mRNA in adipocytes correlates with body weight (7, 8). Furthermore, serum immunoreactive leptin levels show a strong positive correlation with body fat, being elevated in obesity (7, 9). Although the mechanisms by which leptin acts are far from being understood, the presence of leptin receptors suggests that the brain is one of the main loci of leptin's action (10-15). In addition the recent demonstration of leptin receptors in some specific nuclei such as the arcuate or the periventricular nuclei (13-15) provides the basis for a neuroendocrine role of leptin in the control of anterior pituitary hormone secretion.

In order to test the possibility that leptin serves as a metabolic signal that acts on the hypothalamus influencing GH secretion, we studied the effects of immunoneutralization of endogenous leptin, after administration of leptin antibodies, as well as the effect of exogenous leptin on spontaneous GH secretion in freely moving rats.

MATERIAL AND METHODS.

Synthesis of leptin and generation of leptin antibodies.

Recombinant full length human leptin was produced in *Saccharomyces cerevisiae* as described elsewhere (9). Leptin-antibodies were generated in rabbits after administration of full-length mouse leptin as described elsewhere (9).

Animals and experimental procedure.

Adult male Sprague-Dawley rats (200-250 g) were housed on a 12-h light, 12-h dark cycle in a temperature and humidity controlled room. Chronic i.c.v. and intracardiac cannulae were implanted under sodium pentobarbital (50 mg/Kg, ip) anaesthesia, as described previously (16). After surgery, the animals were placed directly in isolation test chambers for 5 days and given free access to regular Purina rat chow and tap water. Thereafter the animals continued to have food available ad libitum or were deprived of food for 48 h before blood sampling. On the day of the experiment, blood samples (0.3 ml) were withdrawn every 15 min for periods of 6 h (10.00-16.00 h) on freely-moving rats. At 10.15h the

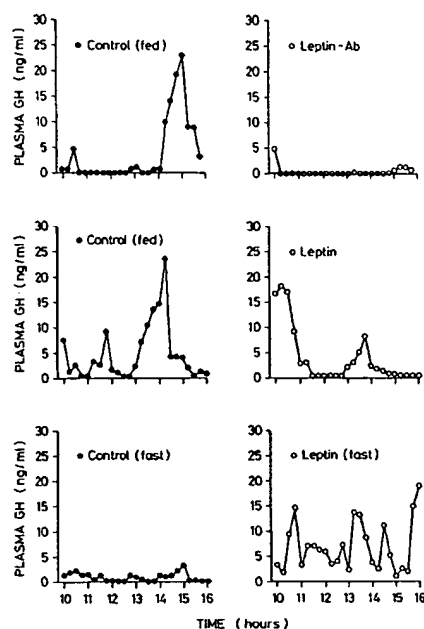


Fig. 1. Representative plasma GH profiles in individual male rats after i.c.v. injection of 10 μ l NRS (control (fed); upper left panel) or 10 μ l of specific antiserum against leptin (Leptin-Ab); 10 μ l of vehicle or 10 μ l containing 10 μ g of leptin in fed rats (middle panel) or fasted rats (lower panel). All the substances or vehicle were administered at 10.15 h. (n = 6 to 8 rats per group).

animals received either normal rabbit serum (NRS), leptin antiserum, vehicle or leptin through the i.c.v. route.

Hormone assays.

Plasma GH concentrations were determined by double antibody RIA using materials supplied by the NHPP. Values are expressed in terms of the GH reference preparation (GH-RP-2).

Statistical analysis.

Assessment of spontaneous GH secretion was carried out as described previously with the

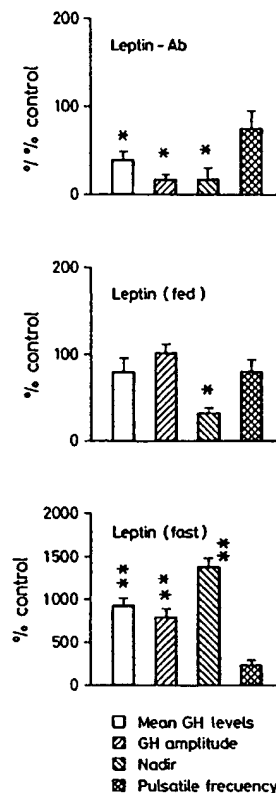


Fig. 2. Characteristics of the GH secretory pattern in male rats injected with leptin antiserum (Leptin-Ab; upper, panel) in fed rats or leptin in fed and fasted rats (middle and lower panel). Values are shown as the mean \pm SE in relation to the appropriate control group. * p < 0.05, ** p < 0.01 (vs NRS or vehicle-treated controls)

ULTRA program (16). Comparison between the different groups was assessed by the Mann-Whitney test.

RESULTS

The normal animals, following administration of NRS, exhibited the typical ultradian GH rhythm with a mean GH amplitude of 14.6 ± 8 ng/ml, mean nadir levels of 1.1 ± 0.35 ng/ml and a total GH secretion as assessed by the area under the curve (AUC) of 813 ± 179 ng/ml/6h. Administration of leptin antiserum to the normal

fed rats was associated to a marked decrease in mean GH amplitude, (2.6 ± 1.8 ng/ml, $p < 0.01$) mean nadir GH levels (0.2 ± 0.1 ng/ml, $p < 0.05$) and AUC (168 ± 72 ng/ml/6h; $p < 0.01$) in comparison to rats that received NRS (Fig 1-2).

Intracerebroventricular leptin administration to the normal fed rats did not modify the pulsatile secretion of GH apart from a slight decrease in mean nadir GH levels (Figs 1,2). In contrast, leptin administration to the food restricted rats, reversed the inhibitory effect exerted by food deprivation on spontaneous GH secretion as assessed by mean GH amplitude (1.9 ± 0.9 ng/ml vs 15.6 ± 4 ng/ml, $p < 0.01$), mean nadir GH levels (0.2 ± 0.1 ng/ml vs 3.5 ± 0.3 ng/ml, $p < 0.05$) and AUC (77 ± 32 ng/ml/6h vs 1650 ± 351 ng/ml/6h, $p < 0.01$) (Figs 1,2).

DISCUSSION

The discovery and anti-obesity effects of leptin appears as a breakthrough in the understanding of adipose tissue regulation and will help to understand the pathophysiology of different clinical entities associated to changes in body weight and body composition. Although the intrinsic mechanisms through which it exerts its effects are far from being completely understood, the accepted working hypothesis at present is that it acts on the brain to reduce food intake as well as to increase energy expenditure (17-20). In addition to the presence of leptin receptors in different hypothalamic nuclei, several studies have reported effects of leptin on the hypothalamic pituitary axis. Leptin administration increased plasma LH, FSH and testosterone in fasted normal mice as well as in ob/ob mice (21-22). Furthermore leptin administration to fasted mice increased thyroxine levels and decreased ACTH and corticosterone levels (21). Taken together this data suggests that leptin can play an important neuroendocrine role in anterior pituitary hormone secretion.

In the present work we found that administration of leptin antiserum to normal fed rats led to a clear-cut decrease in plasma GH levels indicating that physiological leptin levels are needed in order to ensure normal spontaneous GH secretion. On the other hand the lack of effect of exogenous leptin administration in normal fed animals suggests that normal circulating leptin levels are exerting a maximal effect. To further characterize the role of leptin in GH secretion we next studied its effect in fasted animals, an experimental model that is associated to a decrease in circulating leptin levels (21,23).

In agreement with previous data, we have found a marked suppression of GH secretion in response to food deprivation. Furthermore, we

found that following i.c.v. leptin administration there was a reversal of the inhibitory effect exerted by food deprivation on in vivo GH secretion. Taking into account that pituitary GH content and GH responses to exogenously administered GHRH is increased in starved rats, it has been postulated that impaired GH secretion in these animals is due to an alteration at hypothalamic level (23-24). In support of this possibility, a decrease in hypothalamic preproGHRH in starved rats was found (24). Suppression of GH secretion in food-deprived rats is also considered to be at least in part due to increased somatostatin release, because passive immunization with antiserum to somatostatin reverses starvation-induced inhibition of GH secretion (23). The increase in the hypothalamic somatostatinergic tone in food deprived animals is thought to be mediated by neuropeptide Y. Hypothalamic NPY release is increased following food deprivation in rats (25). Leptin receptors are present in NPY producing neurones in the arcuate nuclei (14) and there is immunohistochemical evidence for a synaptic connection between NPY-containing axons and periventricular somatostatin neurones (25). NPY stimulates in vitro somatostatin release and inhibits in vivo GH secretion (25-26). Furthermore, central administration of antiserum to NPY reverses starvation induced-GH release in a similar fashion to that observed following administration of somatostatin antiserum (23,25). This data and the finding that leptin did not modify in vitro GH secretion (the authors unpublished observations) but inhibits in vivo hypothalamic NPY mRNA levels (21) are compatible with the possibility that leptin could serve as a metabolic signal to the GH axis by acting on the brain. Further studies looking at the effects of leptin on NPY, GHRH and somatostatin synthesis and secretion are needed in order to uncover the mechanisms by which it acts.

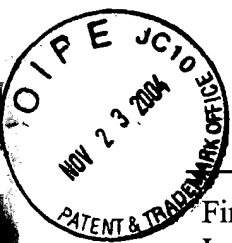
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EXHIBIT P

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DECLARATION

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Involvement of Thyroid Hormones in the Effect of Intracerebroventricular Leptin Infusion on Uncoupling Protein-3 Expression in Rat Muscle

Isabelle Cusin, Juha Rouru, Theo Visser, Albert G. Burger, and Françoise Rohner-Jeanrenaud

We have shown previously that continuous (6 days) intracerebroventricular (ICV) leptin infusion in normal rats resulted in decreases in food intake and body weight. A reduction of food intake imposed on control rats (pair-feeding), aimed at mimicking leptin-induced hyperphagia, produced a marked decrease in the expression of muscle uncoupling protein-3 (UCP-3), whereas ICV infusion of leptin prevented such a decrease in UCP-3. To investigate an involvement of thyroid hormones in this effect of leptin, plasma levels of these hormones were determined in ICV leptin-infused, ICV vehicle-infused ad libitum fed or pair-fed controls. ICV leptin infusion and pair-feeding resulted in decreased plasma thyroid-stimulating hormone (TSH) and T4 levels relative to ad libitum fed controls. ICV leptin infusion maintained plasma levels of T3, but the levels were decreased by pair-feeding. The activity of the enzyme (hepatic 5'-monodeiodinase) responsible for T4/T3 conversion was measured. In the leptin-infused group, the activity of 5'-monodeiodinase was maintained at the values measured in ad libitum fed rats; in pair-fed rats, activity was reduced. Thus, conversion of T4 to T3 is decreased by pair-feeding, whereas such is not the case during leptin infusion. To further substantiate an involvement of thyroid hormones in the effect of leptin on muscle UCP-3 expression, hypothyroid rats were ICV infused with leptin or vehicle. It was observed that in hypothyroid rats, ICV leptin was unable to maintain muscle UCP-3 expression at values measured in ad libitum fed controls. These results suggest that central leptin stimulates T3 production via an activation of T4 to T3 conversion, and that this stimulation could be responsible for the effect of leptin on muscle UCP-3 expression. Thyroid hormones could thus be important mediators of the effect of leptin on energy expenditure. *Diabetes* 49:1101-1105, 2000

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BAT, brown adipose tissue; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; ICV, intracerebroventricular; TSH, thyroid-stimulating hormone; UCP, uncoupling protein; WAT, white adipose tissue.

Leptin is a hormone released by adipose tissue (1). After leptin binds to long-form hypothalamic receptors, it acts as a satiety factor (2-4). Leptin also plays an important role in increasing energy expenditure (4,5), an effect that could be mediated by uncoupling protein (UCP)-1 (6) and by the newly discovered UCP-2 (7) and UCP-3 (8). UCP-1 is abundantly expressed in rodent brown adipose tissue (BAT) and has been shown to have the property of dissipating energy as heat (6). While also expressed in BAT, UCP-2 is present in other tissues, such as white adipose tissue (WAT), lung, liver, and kidney (7). Its role in the heat dissipation process is still a matter of debate. Due to the presence of UCP-3 in both BAT and skeletal muscles, including human muscles (8), UCP-3 is a potential candidate in the regulation of body weight in large mammals, all the more so now that its role in energy dissipation as heat has recently been documented (9).

We have shown previously that chronic central or intravenous leptin infusion is able to maintain or increase the expression of UCPs in different tissues to values observed in ad libitum fed vehicle-infused controls. In contrast, a reduction of food intake imposed on control rats and aimed at mimicking the effect of leptin on this parameter resulted in massive decreases in the expression of the three UCPs (10,11). Such an effect of leptin is in keeping with data by others reporting increases in the expression of UCPs after chronic leptin administration in rats (12-14) or obese mice (15,16).

Thyroid hormones have also been demonstrated to be major regulators of UCPs. T3 treatment increases UCP-3 mRNA in muscle (16,17-19) as well as UCP-2 mRNA in muscle, BAT, and WAT (18,20,21). In contrast, a state of hypothyroidism induces a decrease in the expression of muscle UCP-3 (16,17).

Finally, it has been shown that leptin may modulate the plasma levels of thyroid hormones; as during fasting, leptin treatment prevents the drop of plasma T3 and T4 levels from occurring (22-24).

In view of the above-mentioned considerations, the purpose of the present study was to determine if thyroid hormones could be one of the mediators of the central effect of leptin on muscle UCP-3.

RESEARCH DESIGN AND METHODS

Animals. Eight- to 9-week-old male Sprague-Dawley rats purchased from IFFA Credo (L'Arbresle, France) were housed in individual cages under con-

ditions of controlled temperature (23°C) and illumination (7:00 A.M.–7:00 P.M.). They were allowed ad libitum access to water and standard laboratory diet (Provimi Lacta SA, Cossonay, Switzerland) unless otherwise stated. Food intake and body weight were measured daily.

Chronic intracerebroventricular infusions. Rats were anesthetized with intramuscular ketamin/xylazine used at 45 mg/kg and 9 mg/kg, respectively (Parke-Davis and Bayer, Leverkusen, Switzerland) and equipped with a cannula positioned in the right lateral ventricle. After 1 week of recovery, osmotic minipumps (model 2001, Alza Corporation, Palo Alto, CA) delivering 12.5 µg of leptin per day (recombinant mouse leptin provided by Novartis, Basle, Switzerland) for 6 days or its vehicle (Tris 0.1 mol/l, pH 9) were connected to the intracerebroventricular (ICV) infusion cannula via a polyethylene catheter under ether anesthesia (25). Three groups of rats were investigated: 1 group of rats were ICV infused with leptin; 1 group of control rats were ICV infused with the vehicle and allowed to eat ad libitum; and 1 group of control rats were ICV infused with the vehicle but pair-fed to the amount of food consumed by leptin-infused animals. The pair-feeding regimen was performed as follows: average daily food intake for the leptin-treated group was calculated; one-third of this amount of food was given in the morning (8:00 A.M.), and the remaining two-thirds were given before the extinction of the light (6:00 P.M.), based on a preliminary study of food consumption during the day and the night.

Chemical thyroidectomy. Rats were treated with methimazole (2-mercapto-1-methyl-imidazole; Fluka Chemie, Buchs, Switzerland) in their drinking water at a dose of 0.2 g/l. Eighteen days later, 1 group of rats was ICV infused with leptin for 6 days, and 2 groups of control rats, ad libitum fed or pair-fed respectively, were ICV infused with the vehicle.

5'-Deiodinase type I activity. Monodeiodinase type I activity was determined in liver homogenates using 1 µmol/l rT3 and 1 mmol/l dithiothreitol with 10 min incubation by measuring the release of radioiodine from [¹²⁵I]rT3 according to the method of Leonard and Rosenberg (26).

Northern blots. At the end of each experiment, skeletal muscles were removed and total RNA extracted (27). Aliquots of 10 µg were size-fractionated on 1.5% agarose gels. Blots were hybridized (Quikhyb, Stratagene) to random primed labeled cDNAs for UCP-3 (provided by D. Ricquier, Meudon, France; GenBank Accession U92069), β-actin (Clontech Laboratory, Palo Alto, CA) or glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (Clontech Laboratory) (28). Autoradiographs (X-Omat-AR; Kodak, Rochester, NY) were quantified by densitometry with the Image Quant Software (Molecular Dynamics, Sunnyvale, CA). Abundance of UCP-3 mRNA relative to that of β-actin or G3PDH was expressed as a percentage of corresponding ad libitum fed vehicle-infused controls.

Measurements of plasma hormones and metabolites. Thyroid-stimulating hormone (TSH), T3, and T4 levels were measured by radioimmunoassay (DPC Technic, Los Angeles, CA; Immulite 2000, rat TSH application: LKRTS, T3: L2KT32, T4: L2KT42; Laboratoire de Chimie Clinique, Hôpital Cantonal, Geneva, Switzerland). **Statistical analysis.** Statistical analysis of the data was carried out by 1-way analysis of variance followed by the Turkey procedure for multiple comparisons. The calculations were performed using the Statistica software (Statsoft, Palo Alto, CA). A *P* value < 0.05 was considered statistically significant.

RESULTS

As expected, chronic (6 days) ICV leptin administration in normal rats resulted in a marked decrease in body weight (Table 1). Leptin-treated rats had a 50% decrease in food intake that was mimicked by the pair-feeding regimen (data not shown).

TABLE 1
Effects of ICV leptin administration on body weight changes in normal and hypothyroid rats

	Body weight changes over 6 days (g)
Ad libitum controls	11.7 ± 2.3
Leptin	-26.7 ± 3.3*
Pair-fed controls	-29.9 ± 1.2*
Hypothyroid leptin	-29.6 ± 1.7*
Hypothyroid pair-fed controls	-29.3 ± 4.4*

Data are means ± SE of 5 or 6 animals per group. Continuous vehicle or leptin infusion (12.5 µg/day) over 6 days. **P* ≤ 0.05 compared with ad libitum fed controls.

In euthyroid rats, both the ICV infusion of leptin and the pair-feeding regimen resulted in decreases in plasma TSH levels compared with values obtained in ad libitum fed controls (Fig. 1), although they failed to reach statistical significance. As further shown by Fig. 1, both the ICV leptin infusion and the pair-feeding regimen produced significant decreases in plasma T4 levels (28 and 44%, respectively; intergroup difference, NS) relative to ad libitum fed controls. In contrast, plasma T3 levels of the leptin-infused rats were maintained at values similar to those of ad libitum fed controls, whereas a 28% decrease in the T3 levels was measured in the pair-fed animals (Fig. 1). These results suggested a difference in the conversion of T4 to T3 between the leptin-infused rats and the pair-fed control group. Therefore, the activity and expression of hepatic 5'-monodeiodinase (type I), the main enzyme responsible for T4/T3 conversion, were measured. As shown by Fig. 2, the deiodinase activity was unaltered, but its mRNA levels were decreased by 50% in the leptin-infused group compared with the ad libitum fed one. Quite different was the situation observed in the pair-fed controls, in which the activity of the enzyme was significantly decreased and its expres-

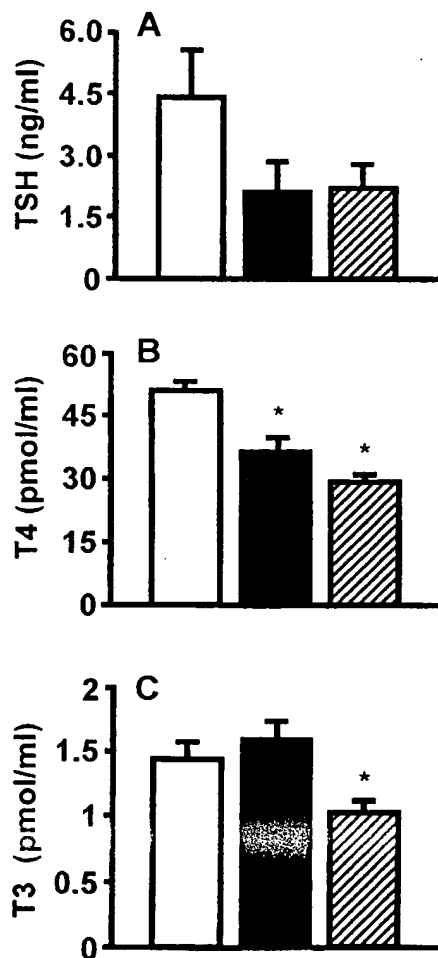


FIG. 1. Plasma TSH (A), T4 (B), and T3 (C) levels in ICV vehicle-infused control rats fed ad libitum (□), ICV leptin-infused rats (■), and ICV vehicle-infused rats pair-fed to the amount of food consumed by the leptin-infused group (▨). Continuous vehicle or leptin infusion (12.5 µg/day) over 6 days. Means ± SE of 5 or 6 animals per group. **P* ≤ 0.01 vs. ad libitum fed controls.

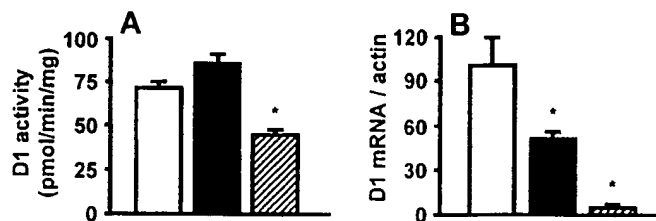


FIG. 2. Monodeiodinase type I (D1) activity (A) and mRNA (B) levels in liver of ICV vehicle-infused control rats fed ad libitum (□), ICV leptin-infused rats (■), and ICV vehicle-infused rats pair-fed to the amount of food consumed by the leptin-infused group (▨). Continuous vehicle or leptin infusion (12.5 µg/day) over 6 days. Means ± SE of 5 or 6 animals per group. * $P \leq 0.05$ vs. ad libitum fed controls.

sion barely detectable (Fig. 2). These results are compatible with the hypothesis that leptin may play a role in maintaining a normal conversion of T4 to T3, a process that is markedly decreased in pair-fed animals by the reduction in food intake mimicking that brought about by leptin.

In earlier experiments, we have shown that leptin infusion to normal rats prevented the decrease in muscle UCP-3 expression that was due to the reduction of food intake elicited by pair-feeding (10). To investigate a possible involvement of thyroid hormones in this effect of leptin, central leptin infusion was carried out in hypothyroid rats, and its effect on muscle UCP-3 expression was compared with that measured in normal ad libitum fed controls and in hypothyroid pair-fed controls. Plasma TSH levels were increased relative to ad libitum fed controls in both groups of hypothyroid rats (147.5 ± 12.2 ng/ml in leptin-infused, 106.7 ± 23.8 ng/ml in pair-fed controls vs. 13.7 ± 2.2 ng/ml in ad libitum fed controls, $n = 5-8$; P at least < 0.05 vs. ad libitum controls). Plasma T3 levels were barely measurable in hypothyroid rats (0.15 ± 0.1 pmol/ml in leptin-infused and 0.48 ± 0.2 pmol/ml in pair-fed control rats, $n = 5-8$, NS), and plasma T4 levels were undetectable in these animals (< 7 pmol/ml). As depicted in Fig. 3, compared with values of muscle UCP-3 mRNA measured in normal ad libitum fed rats, those obtained after a chronic ICV leptin infusion in hypothyroid rats were extremely low and comparable to the values measured in hypothyroid pair-fed controls. Thus, in hypothyroid animals, leptin fails to maintain the expression of UCP-3 as it does in normal rats. The body weight loss brought about by leptin and by pair-feeding in hypothyroid rats was identical to that measured in normal rats (Table 1).

DISCUSSION

We have shown previously that chronic ICV leptin infusion in normal rats maintained or even increased the expression of UCPs in different tissues—that of UCP-3 in muscle, in particular. This was observed in spite of the presence of a decreased food intake produced by the leptin treatment. Food restriction per se (produced by a pair-feeding regimen to mimic the leptin-induced hypophagia) resulted in a marked decrease in the expression of these proteins (10). Such an effect of leptin is in agreement with other studies reporting that leptin administration for several days leads to increased expression of UCP-2 in WAT (12,14) and of UCP-3 in BAT (12) as well as UCP-1 in normal rats when compared with pair-fed rats (12). It is also in keeping with the observa-

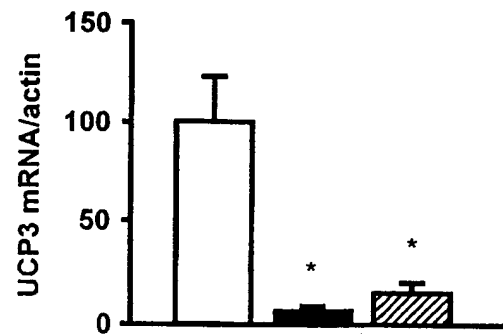


FIG. 3. UCP-3 mRNA levels in ICV vehicle-infused normal rats fed ad libitum (□), hypothyroid ICV leptin-infused rats (■), and hypothyroid ICV vehicle-infused rats pair-fed to the amount of food consumed by the hypothyroid leptin-infused group (▨). Continuous vehicle or leptin infusion (12.5 µg/day) over 6 days. Means ± SE of 5–7 animals per group. * $P \leq 0.01$ vs. ad libitum fed controls.

tion that in the leptin-deficient obese *ob/ob* mouse, UCP-3 expression in skeletal muscle is stimulated by chronic leptin administration (15,16). Noteworthy is the observation that in skeletal muscle of fasted rats, leptin injections do not alter the expression of UCP-3, probably because the latter is already overexpressed by the fasting condition (13,29). Finally, and in contrast to the chronic effects of leptin on the expression of UCPs, when short-term leptin infusion is carried out in normal mice, it has been shown to result in decreases in muscle UCP-3 and WAT UCP-2 expression (30).

The present study was focused on the effect of leptin on muscle UCP-3. Its regulation could be of importance for energy expenditure in large mammals, including humans (31,32), because UCP-3 is the UCP subtype present in skeletal muscle. More specifically, it was hypothesized that thyroid hormones could play a role as mediators of the central effect of leptin on muscle UCP-3 expression.

It was observed that the reduction of food intake per se (pair-feeding regimen), as well as the ICV leptin-induced hypophagia, resulted in decreases in plasma TSH and T4 levels. In contrast, whereas plasma T3 levels were decreased by pair-feeding, they were maintained at normal levels after the central infusion of leptin. This suggested that centrally administered leptin could bring about an increased conversion of T4 to T3. Such a possibility was supported by the observation that central leptin infusion prevented the drop in activity of hepatic 5' monodeiodinase type I observed in pair-fed rats, maintaining such an activity at a similar level to that measured in ad libitum fed controls. In contrast, 5' monodeiodinase type I mRNA levels were lower in the leptin-infused rats than in the ad libitum fed controls, whereas they were barely detectable in the pair-fed control animals. The difference in the effect of leptin on 5' monodeiodinase type I activity and mRNA suggests the existence of some posttranscriptional regulation. The effect of central leptin on thyroid hormones in normal rats is in keeping with results obtained by others showing that leptin prevents the drop in expression of proTSH in the paraventricular nucleus (23), as well as in plasma T3 and T4 levels measured during fasting, maintaining these parameters at normal values (22–24).

As mentioned previously, thyroid hormones have been shown to influence the expression of UCPs. In particular, UCP-3 expression is markedly decreased in skeletal muscle

in hypothyroid animals (16,17), whereas T3 administration to normal rats produces an increase in muscle UCP-3 expression (17–19). The expression of UCP-2 is also increased by T3 treatment in skeletal muscle, heart, cardiomyocytes, BAT, and WAT (18,20,21,33). Furthermore, administration of an inhibitor of the type II 5'-deiodinase leads to a decreased UCP-1 expression in BAT (34).

These data as well as those of the present study suggest that the maintenance of normal hepatic 5'-monodeiodinase activity and of T3 levels produced by the central administration of leptin (as opposed to the decreases thereof in the pair-fed controls) could be essential for the centrally elicited effects of leptin on muscle UCP-3 expression observed in this and in our previous study (10). This contention is supported by the observation that in hypothyroid rats, the ICV infusion of leptin is not able to maintain or stimulate muscle UCP-3 expression, which remains at a low level similar to that observed in pair-fed rats. Thus, thyroid hormones appear to be necessary for the central action of leptin on muscle UCP-3 expression, although other additional factors could be implicated as well. Among those, the sympathetic nervous system is known to be activated by the presence of leptin in the hypothalamus (35,36), and the effects of leptin on glucose metabolism seem to be dependent on the sympathetic tone (37,38). Also, it has been reported that UCP-3 expression is decreased in BAT when catecholamine synthesis is blocked (39) and is no more responsive to leptin in denervated muscles (30). Free fatty acids represent another candidate that may mediate the central effects of leptin on muscle UCP-3. Indeed, they seem to be important regulators of muscle UCP-3 expression (40,41) as, in fasting, UCP-3 expression in skeletal muscle is increased in correlation with the presence of high plasma free fatty acid levels.

Of note is the observation that leptin is able to decrease body weight in hypothyroid rats as it does in normal animals. The effect of leptin on muscle UCP-3 may therefore be important for preventing the rebound of body weight upon normalization of food intake rather than for the actual leptin-induced body weight loss.

In conclusion, the results of the present study strongly suggest that thyroid hormones are among the important mediators of the central effects of leptin on muscle UCP-3 expression. They also suggest that the effects of leptin on energy expenditure may depend on thyroid hormones.

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